TITLE: A METHOD OF USING G-
MATRIX FOURIER
TRANSFORMATION
NUCLEAR MAGNETIC
RESONANCE (GFT NMR)
SPECTROSCOPY FOR RAPID
CHEMICAL SHIFT
ASSIGNMENT AND
SECONDARY STRUCTURE
DETERMINATION OF
PROTEINS

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DOCKET: 19226/2191 (R-5770)

A METHOD OF USING G-MATRIX FOURIER TRANSFORMATION NUCLEAR MAGNETIC RESONANCE (GFT NMR) SPECTROSCOPY FOR RAPID CHEMICAL SHIFT ASSIGNMENT AND SECONDARY STRUCTURE DETERMINATION OF PROTEINS

[0001]   The present invention claims the benefit of U.S. Provisional Patent Application Serial Nos. 60/395,591, filed July 11, 2002, and 60/441,385, filed January 16, 2003, which are hereby incorporated by reference in their entirety. This invention arose out of research sponsored by the National Science Foundation (Grant No. MCB 0075773) and National Institutes of Health (Grant No. P50 GM62413-01). The U.S. Government may have certain rights in this invention.

FIELD OF THE INVENTION

[0002]   The present invention relates to methods of using G-matrix Fourier transformation nuclear magnetic resonance (GFT NMR) spectroscopy for rapidly obtaining and connecting precise chemical shift values and determining the structure of proteins and other molecules.

BACKGROUND OF THE INVENTION

[0003]   Nuclear magnetic resonance (NMR) (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987); Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986); Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996))-based structural studies rely on two broad classes of experimental radio-frequency pulse schemes for recording two-dimensional (2D) (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987)), three-dimensional (3D) (Oschkinat et al., Nature, 332:374-376 (1988)), or four-dimensional (4D) (Kay et al., Science, 249:411-414 (1990)) Fourier transformation (FT) NMR spectra. Correlation spectroscopy (COSY) delineates exclusively scalar coupling connectivities to measure chemical shifts, and (heteronuclear resolved) $^1H,^1H$-nuclear

Overhauser enhancement spectroscopy (NOESY) reveals the strength of through-space dipolar couplings of ^1H spins to estimate distances (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987); Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986)).

NMR spectra need to exhibit (i) signal-to-noise (S/N) ratios warranting reliable data interpretation, (ii) digital resolutions ensuring adequate precision for the measurement of NMR parameters such as chemical shifts, and (iii) a dimensionality at which a sufficient number of NMR parameters is correlated (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987); Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996)). While increased intensity of NOESY peaks ensures their more accurate integration (which, in turn, may translate into increased accuracy of the NMR structure), the mere identification of COSY peaks suffices to obtain the desired chemical shifts. Hence, COSY peak signal-to noise ratios larger that ~3:1 reflect, in essence, inappropriately long measurement times. Moreover, the total number of peaks in COSY grows only linearly with the number of spins involved and is, for a defined magnetization transfer pathway, "independent" of the dimensionality N. Thus, a minimal "target dimensionality" N_t at which most of the COSY peaks detected for a given molecule are resolved can be defined. Further increased dimensionality does not aim at resolving peak overlap but at increasing the number of correlations obtained in a single data set. This eliminates ambiguities when several multidimensional NMR spectra are combined for resonance assignment, for example, when using $^1H, ^{13}C, ^{15}N$ triple-resonance NMR to assign protein resonances (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996)).

[0004] An increase in dimensionality is, however, limited by the need to independently sample the indirect dimensions, because this leads to longer measurement times. Although the measurement time can be somewhat reduced by aliasing signals (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996)) or accepting a lower digital resolution in the indirect dimensions, high dimensionality often prevents one from tuning the measurement time to a value that ensures to obtain sufficient, but not unnecessarily large S/N ratios.

[0005] In view of these considerations, "sampling" and "sensitivity limited" data collection regimes are defined (Szyperski et al., Proc. Natl. Acad. Sci. USA,

99:8009-8014 (2002)), depending on whether the sampling of the indirect dimensions or the sensitivity of the FT NMR experiment determines the minimal measurement time. In the sensitivity limited regime, long measurement times are required to achieve sufficient S/N ratios, so that the sampling of indirect dimensions is not necessarily constraining the adjustment of the measurement time. In the sampling limited regime, some or even most of the instrument time is invested for sampling, which yields excessively large S/N ratios. In view of the ever increasing sensitivity of NMR instrumentation, new methodology to avoid the sampling limited regime is needed. (Szyperski et al., Proc. Natl. Acad. Sci. USA, 99:8009-8014 (2002)).

[0006]   In general, phase-sensitive acquisition of an N-dimensional (ND) FT NMR experiment (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987); Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996)) requires sampling of N-1 indirect dimensions with n_1 x n_2 ... n_{N-1} complex points representing $n_{FID} = 2^{N-1} \cdot \prod_{j=1}^{N-1} n_j$ free induction decays (FIDs). The resulting steep increase of the minimal measurement time, T_m, with dimensionality prevents one from recording five- or higher-dimensional FT NMR spectra: acquiring 16 complex points in each indirect dimension (with one scan per FID each second) yields $T_m(3D) = 0.5$ hour, $T_m(4D) = 9.1$ hours, $T_m(5D) = 12$ days, and $T_m(6D) = 1.1$ years.

[0007]   Thus, higher-dimensional FT NMR spectroscopy suffers from two major drawbacks: (i) The minimal measurement time of an ND FT NMR experiment, which is constrained by the need to sample N-1 indirect dimensions, may exceed by far the measurement time required to achieve sufficient signal-to-noise ratios. (ii) The low resolution in the indirect dimensions severely limits the precision of the indirect chemical shift measurements.

[0008]   The present invention is directed to overcoming the deficiencies in the art.

SUMMARY OF THE INVENTION

[0009]   The present invention relates to a method of conducting a (N,N-K) dimensional (D) G-matrix Fourier transformation (GFT) nuclear magnetic resonance

(NMR) experiment, where N is the dimensionality of an N-dimensional (ND) Fourier transformation (FT) NMR experiment and K is the desired reduction in dimensionality relative to N. The method involves providing a sample and applying radiofrequency pulses for the ND FT NMR experiment to the sample. Then, m indirect chemical shift evolution periods of the ND FT NMR experiment are selected, where m equals K+1, and the m indirect chemical shift evolution periods are jointly sampled. Next, NMR signals detected in a direct dimension are independently cosine and sine modulated to generate $(N\text{-}K)$D basic NMR spectra containing frequency domain signals with 2^K chemical shift multiplet components, thereby enabling phase-sensitive sampling of all jointly sampled m indirect chemical shift evolution periods. Finally, the $(N\text{-}K)$ D basic NMR spectra are transformed into $(N\text{-}K)$ D phase-sensitively edited basic NMR spectra, where the 2^K chemical shift multiplet components of the $(N\text{-}K)$ D basic NMR spectra are edited to yield $(N\text{-}K)$ D phase-sensitively edited basic NMR spectra having individual chemical shift multiplet components.

[0010]   Another aspect of the present invention relates to a method for sequentially assigning chemical shift values of an α-proton, $^1\mathrm{H}^\alpha$, an α-carbon, $^{13}\mathrm{C}^\alpha$, a polypeptide backbone carbonyl carbon, $^{13}\mathrm{C}'$, a polypeptide backbone amide nitrogen, $^{15}\mathrm{N}$, and a polypeptide backbone amide proton, $^1\mathrm{H}^\mathrm{N}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,2)D [HACACONHN] GFT NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i-1, $^1\mathrm{H}^\alpha_{i\text{-}1}$, the α-carbon of amino acid residue i-1, $^{13}\mathrm{C}^\alpha_{i\text{-}1}$, the polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}\mathrm{C}'_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}\mathrm{N}_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1\mathrm{H}^\mathrm{N}_i$ and (2) a (5,2)D [HACA,CONHN] GFT NMR experiment to measure and connect the chemical shift values of $^1\mathrm{H}^\alpha_{i\text{-}1}$, $^{13}\mathrm{C}^\alpha_{i\text{-}1}$, $^{13}\mathrm{C}'_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}\mathrm{N}_{i\text{-}1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^1\mathrm{H}^\mathrm{N}_{i\text{-}1}$. Then, sequential assignments of the chemical shift values of $^1\mathrm{H}^\alpha$, $^{13}\mathrm{C}^\alpha$, $^{13}\mathrm{C}'$, $^{15}\mathrm{N}$, and $^1\mathrm{H}^\mathrm{N}$ are obtained by (i) matching the chemical shift values of $^1\mathrm{H}^\alpha_{i\text{-}1}$, $^{13}\mathrm{C}^\alpha_{i\text{-}1}$, and $^{13}\mathrm{C}'_{i\text{-}1}$ measured by the

(5,2)D [HACACONHN] GFT NMR experiment with the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,2)D [HACA,CONHN] GFT NMR experiment, (ii) using the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0011]   Yet another aspect of the present invention relates to a method for sequentially assigning chemical shift values of an α-proton, $^1H^{\alpha}$, an α-carbon, $^{13}C^{\alpha}$, a polypeptide backbone carbonyl carbon, $^{13}C'$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,3)D [HACACONHN] GFT NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i-1, $^1H^{\alpha}_{i-1}$, the α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$, the polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$ and (2) a (5,3)D [HACA,CONHN] GFT NMR experiment to measure and connect the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^1H^N_{i-1}$. Then, sequential assignments of the chemical shift values of $^1H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C'$, ^{15}N, and $^1H^N$ are obtained by (i) matching the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,3)D [HACACONHN] GFT NMR experiment with the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,3)D [HACA,CONHN] GFT NMR experiment, (ii) using the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0012] A further aspect of the present invention relates to a method for sequentially assigning chemical shift values of α- and β-carbons, $^{13}C^{\alpha/\beta}$, a polypeptide backbone carbonyl carbon, $^{13}C'$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^{1}H^{N}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (4,3)D [CBCACONHN] GFT NMR experiment to measure and connect the chemical shift values of the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i\text{-}1}$, the polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_{i}$, and the polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_{i}$ and (2) a (4,3)D [CBCA,CONHN] GFT NMR experiment to measure and connect the chemical shift values of $^{13}C^{\alpha/\beta}_{i\text{-}1}$, $^{13}C'_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i\text{-}1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^{1}H^{N}_{i\text{-}1}$. Then, sequential assignments of the chemical shift values of $^{13}C^{\alpha/\beta}$, $^{13}C'$, ^{15}N, and $^{1}H^{N}$ are obtained by (i) matching the chemical shift values of $^{13}C^{\alpha/\beta}_{i\text{-}1}$ and $^{13}C'_{i\text{-}1}$ measured by the (4,3)D [CBCACONHN] GFT NMR experiment with the chemical shift values of $^{13}C^{\alpha/\beta}_{i\text{-}1}$ and $^{13}C'_{i\text{-}1}$ measured by the (4,3)D [CBCA,CONHN] GFT NMR experiment, (ii) using the chemical shift values of $^{13}C^{\alpha/\beta}_{i\text{-}1}$ and $^{13}C'_{i\text{-}1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0013] The present invention also relates to a method for sequentially assigning chemical shift values of α- and β-carbons, $^{13}C^{\alpha/\beta}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^{1}H^{N}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (4,3)D [HNNCACBCA] GFT NMR experiment to measure and connect the chemical shift values of the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i\text{-}1}$, the α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i\text{-}1}$, and the

polypeptide backbone amide proton of amino acid residue i-1, $^1\text{H}^N_{i\text{-}1}$ and (2) a GFT NMR experiment selected from the group consisting of a (4,3)D [HNN(CO)<u>CACBCA</u>] GFT NMR experiment, a (4,3)D [<u>CBCACA</u>(CO)NHN] GFT NMR experiment , and a (5,3)D [<u>HBHACBCACA</u>(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of $^{13}\text{C}^{\alpha/\beta}_{i\text{-}1}$, $^{13}\text{C}^{\alpha}_{i\text{-}1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}\text{N}_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1\text{H}^N_i$. Then, sequential assignments of the chemical shift values of $^{13}\text{C}^{\alpha/\beta}$, ^{15}N, and $^1\text{H}^N$ are obtained by (i) matching the chemical shift values of $^{13}\text{C}^{\alpha/\beta}_{i\text{-}1}$ measured by the GFT NMR experiment selected from the group consisting of a (4,3)D [HNN(CO)<u>CACBCA</u>] GFT NMR experiment, a (4,3)D [<u>CBCACA</u>(CO)NHN] GFT NMR experiment , and a (5,3)D [<u>HBHACBCACA</u>(CO)NHN] GFT NMR experiment with the chemical shift values of $^{13}\text{C}^{\alpha/\beta}_{i\text{-}1}$ measured by the (4,3)D [HNN<u>CACBCA</u>] GFT NMR experiment, (ii) using the chemical shift values of $^{13}\text{C}^{\alpha/\beta}_{i\text{-}1}$ to identify the type of amino acid residue i-1, and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements within the polypeptide chain.

[0014] Another aspect of the present invention relates to a method for assigning chemical shift values of γ-, δ-, and ε-aliphatic sidechain protons, $^1\text{H}^{\gamma/\delta/\varepsilon}$, and chemical shift values of γ-, δ-, and ε-aliphatic sidechain carbons located peripheral to β-carbons, $^{13}\text{C}^{\gamma/\delta/\varepsilon}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,3)D [<u>HCC</u>,CH-COSY] GFT NMR experiment to measure and connect the chemical shift values of a proton of amino acid residue i-1, $^1\text{H}_{i\text{-}1}$, a carbon of amino acid residue i-1 coupled to $^1\text{H}_{i\text{-}1}$, $^{13}\text{C}_{i\text{-}1}$, a carbon coupled to $^{13}\text{C}_{i\text{-}1}$, $^{13}\text{C}_{i\text{-}1}^{\text{coupled}}$, and a proton coupled to $^{13}\text{C}_{i\text{-}1}^{\text{coupled}}$, $^1\text{H}_{i\text{-}1}^{\text{coupled}}$, and (2) a (5,3)D [<u>HBHACBCACA</u>(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of α- and β-protons of amino acid residue i-1, $^1\text{H}^{\alpha/\beta}_{i\text{-}1}$, and α- and β-carbons of amino acid residue i-1, $^{13}\text{C}^{\alpha/\beta}_{i\text{-}1}$. Then, assignments of the chemical shift values of $^1\text{H}^{\gamma/\delta/\varepsilon}$ and $^{13}\text{C}^{\gamma/\delta/\varepsilon}$ are obtained by (i) identifying $^1\text{H}_{i\text{-}1}$, $^{13}\text{C}_{i\text{-}1}$, $^{13}\text{C}_{i\text{-}1}^{\text{coupled}}$, and $^1\text{H}_{i\text{-}1}^{\text{coupled}}$ measured by the (5,3)D [<u>HCC</u>,CH-COSY] GFT NMR experiment as $^1\text{H}^{\alpha}_{i\text{-}1}$, $^{13}\text{C}^{\alpha}_{i\text{-}1}$, $^{13}\text{C}^{\beta}_{i\text{-}1}$, and $^1\text{H}^{\beta}_{i\text{-}}$

1, respectively, and thereby matching the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ with the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ measured by the (5,3)D HBHACBCACA(CO)NHN] GFT NMR experiment, and (ii) using the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ in conjunction with other chemical shift connections from the (5,3)D [HCC,CH-COSY] GFT NMR experiment to measure the chemical shift values of $^1H^{\gamma/\delta/\epsilon}_{i-1}$ and $^{13}C^{\gamma/\delta/\epsilon}_{i-1}$.

[0015]   Yet another aspect of the present invention relates to a method for assigning chemical shift values of γ-, δ-, and ϵ-aliphatic sidechain protons, $^1H^{\gamma/\delta/\epsilon}$, and chemical shift values of γ-, δ-, and ϵ-aliphatic sidechain carbons located peripheral to β-carbons, $^{13}C^{\gamma/\delta/\epsilon}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (4,2)D [HCCH-COSY] GFT NMR experiment to measure and connect the chemical shift values of a proton of amino acid residue i-1, $^1H_{i-1}$, a carbon of amino acid residue i-1 coupled to $^1H_{i-1}$, $^{13}C_{i-1}$, a carbon coupled to $^{13}C_{i-1}$, $^{13}C_{i-1}^{coupled}$, and a proton coupled to $^{13}C_{i-1}^{coupled}$, $^1H_{i-1}^{coupled}$, and (2) a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of α- and β-protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$, and α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$. Then, assignments of the chemical shift values of $^1H^{\gamma/\delta/\epsilon}$ and $^{13}C^{\gamma/\delta/\epsilon}$ are obtained by (i) identifying $^1H_{i-1}$, $^{13}C_{i-1}$, $^{13}C_{i-1}^{coupled}$, and $^1H_{i-1}^{coupled}$ measured by the (4,2)D [HCCH-COSY] GFT NMR experiment as $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C^{\beta}_{i-1}$, and $^1H^{\beta}_{i-1}$, respectively, and thereby matching the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ with the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ measured by the (5,3)D HBHACBCACA(CO)NHN] GFT NMR experiment, and (ii) using the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ in conjunction with other chemical shift connections from the (4,2)D [HCCH-COSY] GFT NMR experiment to measure the chemical shift values of $^1H^{\gamma/\delta/\epsilon}_{i-1}$ and $^{13}C^{\gamma/\delta/\epsilon}_{i-1}$.

[0016]   A further aspect of the present invention relates to a method for assigning chemical shift values of a γ-carbon, $^{13}C^{\gamma}$, a δ-carbon, $^{13}C^{\delta}$, and a δ-proton, $^1H^{\delta}$, of an amino acid residue containing an aromatic spin system in a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments

on the protein sample including: (1) a (5,3)D [<u>HBCB</u>CG<u>CD</u>HD] GFT NMR experiment to measure and connect the chemical shift values of a β-proton of amino acid residue i-1, $^1H^\beta{}_{i\text{-}1}$, a β-carbon of amino acid residue i-1, $^{13}C^\beta{}_{i\text{-}1}$, a γ-carbon of amino acid residue i-1, $^{13}C^\gamma{}_{i\text{-}1}$, a δ-carbon of amino acid residue i-1, $^{13}C^\delta{}_{i\text{-}1}$, and a δ-proton of amino acid residue i-1, $^1H^\delta{}_{i\text{-}1}$, and (2) a (5,3)D [<u>HBHACBCACA</u>(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of $^1H^\beta{}_{i\text{-}1}$ and $^{13}C^\beta{}_{i\text{-}1}$. Then, assignments of the chemical shift values of $^{13}C^\gamma$, $^{13}C^\delta$, and $^1H^\delta$ are obtained by (i) matching the chemical shift values of $^1H^\beta{}_{i\text{-}1}$ and $^{13}C^\beta{}_{i\text{-}1}$ measured by the (5,3)D <u>HBCBCACA</u>(CO)NHN GFT NMR experiment with the chemical shift values of $^1H^\beta{}_{i\text{-}1}$ and $^{13}C^\beta{}_{i\text{-}1}$ measured by the (5,3)D [<u>HBCB</u>CG<u>CD</u>HD] GFT NMR experiment, and (ii) using the chemical shift values of $^{13}C^\gamma$, $^{13}C^\delta$, and $^1H^\delta$ to identify the type of amino acid residue containing the aromatic spin system.

[0017] The present invention also relates to a method for assigning chemical shift values of aliphatic and aromatic protons and aliphatic and aromatic carbons of an amino acid residue containing aliphatic and aromatic spin systems in a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a first GFT NMR experiment, which is selected from the group consisting of a (5,3)D [<u>HCC</u>,CH-COSY] GFT NMR experiment, a (4,2)D [<u>HCC</u>H-COSY] GFT NMR experiment, a (5,2)D [<u>HCCC</u>H-COSY] GFT NMR experiment, and a (5,3)D [<u>HCCC</u>H-COSY] GFT NMR experiment and is acquired for the aliphatic spin system, to measure and connect the chemical shift values of α- and β-protons of amino acid residue i, $^1H^{\alpha/\beta}{}_i$, α- and β-carbons of amino acid residue i, $^{13}C^{\alpha/\beta}{}_i$, a γ-carbon of amino acid residue i, $^{13}C^\gamma{}_i$, and (2) a second GFT NMR experiment, which is selected from the group consisting of a (5,3)D [<u>HCC</u>,CH-COSY] GFT NMR experiment, a (4,2)D [<u>HCC</u>H-COSY] GFT NMR experiment, a (5,2)D [<u>HCCC</u>H-COSY] GFT NMR experiment, and a (5,3)D [<u>HCCC</u>H-COSY] GFT NMR experiment and is acquired for the aromatic spin system, to measure and connect the chemical shift values of $^{13}C^\gamma{}_i$ and other aromatic protons and carbons of amino acid residue i. Then, assignments of the chemical shift values of the aliphatic and aromatic protons and aliphatic and aromatic carbons are obtained by matching

the chemical shift value of $^{13}C^{\gamma}_i$ measured by the first GFT NMR experiment with the chemical shift value of $^{13}C^{\gamma}_i$ measured by the second GFT NMR experiment.

[0018] Another aspect of the present invention relates to a method for obtaining assignments of chemical shift values of ^{1}H, ^{13}C, and ^{15}N of a protein molecule. The method involves providing a protein sample and conducting five G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample, where (1) a first experiment is a (4,3)D [HNNCACBCA] GFT NMR experiment for obtaining intraresidue correlations of chemical shift values; (2) a second experiment is a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment for obtaining interresidue correlations of chemical shift values; (3) a third experiment is a (5,3)D [HCC,CH-COSY] GFT NMR experiment for obtaining assignments of aliphatic sidechain chemical shift values; (4) a fourth experiment is a (5,3)D [HBCBCGCDHD] GFT NMR experiment for linking chemical shift values of aliphatic protons, $^{1}H^{\beta}$ and $^{13}C^{\beta}$, and aromatic protons, $^{13}C^{\delta}$ and $^{1}H^{\delta}$; and (5) a fifth experiment is a (4,2)D [HCCH-COSY] GFT NMR experiment for obtaining assignments of aromatic sidechain chemical shift values.

[0019] The present invention discloses a number of specific GFT NMR experiments and different combinations of those experiments which allows one to obtain sequential backbone chemical shift assignments for determining the secondary structure of a protein molecule and complete assignments of chemical shift values for a protein molecule including aliphatic and aromatic sidechain spin systems.

[0020] The present invention provides a generally applicable approach for NMR data acquisition and processing named "GFT NMR spectroscopy". This approach is based on the phase-sensitive joint sampling of several indirect dimensions while ensuring that all chemical shift correlations are retained. The employment of GFT NMR focuses on the sampling limited data collection regime and, considering that NMR measurements longer than about a week are impracticable, on the acquisition of five- or higher-dimensional spectral information.

[0021] GFT NMR relaxes on constraints arising from two major drawbacks of FT NMR, that is, the problem of having excessive or prohibitively long measurement times due to sampling of indirect dimensions and the limited precision of chemical shift measurements in the indirect dimensions arising from comparably low digital

resolution. Within a few hours or less, GFT NMR spectroscopy affords the correlations of even five- or higher-dimensional FT NMR spectra acquired with high digital resolution. Thus, GFT NMR spectroscopy allows one to tune measurement times to sensitivity requirements without compromising on the dimensionality or the digital resolution. High-throughput efforts such as NMR-based structural genomics (Montelione et al., Nat. Struct. Biol., 7:982-984 (2000), which is hereby incorporated by reference in its entirety) will profit from this feature, because automated resonance assignment (Szyperski et al., J. Biomol. NMR, 11:387-405 (1998); Moseley et al, Curr. Opin. Struct. Biol., 9:635-642 (1999); Moseley et al., Methods Enzymol., 339:91-108 (2001), which are hereby incorporated by reference in their entirety) benefits from maximizing the number of correlations obtained from in a single NMR experiment. Moreover, the rapid sampling realized with GFT NMR spectroscopy will allow researchers to obtain highest dimensional NMR information with exceptional time resolution when, for example, studying slow protein folding in real time (Dyson et al., Annu. Rev. Phys. Chem., 47:369-395 (1996), which is hereby incorporated by reference in its entirety). The high precision of the chemical shift measurements is of potential importance for a broad range of NMR applications in natural sciences and engineering, for example, for automated assignment, or when studying systems with high chemical shift degeneracy such as RNA ribose spin systems (Cromsigt et al., Methods Enzymol., 338:371-399 (2001), which is hereby incorporated by reference in its entirety), (partially) unfolded proteins (Neri et al., FEBS Lett., 303:129-135 (1992), which is hereby incorporated by reference in it its entirety), or lipids (Wang et al., Biochemistry, 41:5453-5461 (2002), which is hereby incorporated by reference in its entirety). Finally, the high precision of the shift measurements may be recruited to accurately measure other NMR parameters such as residual dipolar couplings for structural refinement (Tjandra et al., Science, 278:1111-1114 (1997); Prestegard, Nat. Struct. Biol., 5:517-522 (1998), which are hereby incorporated by reference in their entirety), and transverse relaxation optimized (Pervushin et al., Proc. Natl. Acad. Sci. USA, 94:12366-12371 (1997), which is hereby incorporated by reference in its entirety) GFT NMR may develop into a powerful approach to investigate larger systems.

[0022]   In the sensitivity limited regime, GFT NMR may be advantageous in cases where an extended radiofrequency (rf) phase cycle is desirable for spectral

editing and/or improved artifact suppression (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety).

BRIEF DESCRIPTION OF THE DRAWINGS

[0023]   Figure 1 compares the conventional sampling of a 3D time domain subspace of an ND FT NMR experiment (on the left) with the phase-sensitive joint sampling of the three dimensions in an $(N,N-2)$D GFT NMR (on the right), that is, with $K = 2$. Processing of the FT NMR experiment requires a 3D FT of the subspace, while the GFT NMR experiment requires time domain editing of chemical shift multiplet components by application of the so-called G-matrix (see equation 1 in the "Detailed Description of the Invention") and 1D FT of the resulting $p = 2^{K+1}$ data sets. For the GFT NMR experiment, the phase settings of ϕ_1 and ϕ_2 of the rf pulses creating transverse magnetization for frequency labeling with Ω_1 and Ω_2 are indicated for basic spectra (top four rows), first-order central peak spectra (two rows in the middle), and the second-order central peak spectrum (bottom row). Instead of a single peak in FT NMR which encodes three chemical shifts, one obtains a p-fold overdetermined system of equations. A least-squares fit calculation yields the three shifts from the position of seven peaks. In a GFT NMR experiment with constant-time chemical shift evolution periods, the lines forming the chemical shift multiplets have the same width as the resonances in FT NMR (if recorded with corresponding maximal evolution times; see also Figures 18A-B). (The width at half height of the frequency domain sinc centre lobe resulting from truncation in the time domain at t_{max} is given (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) by $0.604/t_{max}$. In the current implementation of (5,2)D HACACONHN (Figure 6) all indirect evolution periods except for $\Omega(^1H^\alpha)$ are constant-time periods. The evolution of $\Omega(^1H^\alpha)$ is implemented in a semiconstant-time manner (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety), so that signal losses due to transverse relaxation of $^1H^\alpha$ are negligible for 8.6 kDa ubiquitin at short t_{max} values around 6.5

ms. For larger systems with short $T_2(^1H^\alpha)$, however, the semiconstant-time frequency labeling may lead to a detectable increase of ω_1-line widths in the basic when compared to central peak spectra.) This yields the same standard deviation $\Delta\Omega$ for the identification of peak positions in the two experiments. Hence, the standard deviation of the chemical shift measurements obtained "after" the least-squares fit is reduced (Eadie et al., Statistical Methods in Experimental Physics, North-Holland, New York (1982), which is hereby incorporated by reference in its entirety) by a factor $1/\sqrt{n}$ in GFT NMR. For simplicity, it is assumed that the n peaks which contribute to the calculation of a given shift exhibit the same line widths (see descriptions of Figures 19-20).

[0024] Figures 2A-D show a stick diagram exemplifying the formation of chemical shift multiplets (on the left) for $K = 3$ and phase-sensitively edited multiplet components (on the right) in the frequency domain. Figure 2A shows the basic spectra yielding the following linear combinations: $B1[\Omega_0 + \Omega_1 + \Omega_2 + \Omega_3] = A1 + A2 + A3 + A4 + A5 + A6 + A7 + A8$; $B2[\Omega_0 - \Omega_1 + \Omega_2 + \Omega_3] = A1 - A2 + A3 - A4 + A5 - A6 + A7 - A8$; $B3[\Omega_0 + \Omega_1 - \Omega_2 + \Omega_3] = A1 + A2 - A3 - A4 + A5 + A6 - A7 - A8$; $B4[\Omega_0 - \Omega_1 - \Omega_2 + \Omega_3] = A1 - A2 - A3 + A4 + A5 - A6 - A7 + A8$; $B5[\Omega_0 + \Omega_1 + \Omega_2 - \Omega_3] = A1 + A2 + A3 + A4 - A5 - A6 - A7 - A8$; $B6[\Omega_0 - \Omega_1 + \Omega_2 - \Omega_3] = A1 - A2 + A3 - A4 - A5 + A6 - A7 + A8$; $B7[\Omega_0 + \Omega_1 - \Omega_2 - \Omega_3] = A1 + A2 - A3 - A4 - A5 - A6 + A7 + A8$; $B8[\Omega_0 - \Omega_1 - \Omega_2 - \Omega_3] = A1 - A2 - A3 + A4 - A5 + A6 + A7 - A8$. Figure 2B shows the first order central peak spectra: $B9[\Omega_0 + \Omega_1 + \Omega_2] = A9 + A10 + A11 + A12$; $B10[\Omega_0 - \Omega_1 + \Omega_2] = A9 - A10 + A11 - A12$; $B11[\Omega_0 + \Omega_1 - \Omega_2] = A9 + A10 - A11 - A12$; $B12[\Omega_0 - \Omega_1 - \Omega_2] = A9 - A10 - A11 + A12$. Figure 2C shows the second order central peak spectra: $B13[\Omega_0 + \Omega_1] = A13 + A14$; $B14[\Omega_0 - \Omega_1] = A13 - A14$. Figure 2D shows the third order central peak spectra: $B15 = A15$. For the calculation of the matrices $F(K)$, see Example 1. To facilitate the comparison of the left and the right section the positions of multiplet components are indicated with thin lines.

[0025] Figures 3A-B illustrate the "bottom-up" identification of the peaks forming a chemical shift multiplet in GFT NMR, provided that three indirect dimensions of a FT NMR experiment are jointly sampled (Figure 1; $K = 2$). Figure 3A shows that two spin systems exhibiting degenerate chemical shifts in all other conventionally sampled $N_t - 1$ dimensions give rise to basic, first order central and

second order central peaks shown in bold (spin system 1) and lighter shade (spin system 2), respectively. Knowledge of the position of the second order central peak of spin system 1 allows identification of the corresponding first order central peaks of spin system 1. In turn, their knowledge allows unambiguous identification of the corresponding peaks of spin system 1 in the basic spectra. As indicated by the dashed line on the left in Figure 3A, the peaks in $B1$ and $B3$ (shown in bold) are centered around the peak in $B5$ (shown in bold), while, as indicated by the dashed line on the right in Figure 3A, the peaks in $B2$ and $B4$ (shown in bold) are centered around the peak in $B5$ (shown in bold). This strategy can readily be extended for $K > 2$. In practice, the identification of components belonging to a given shift multiplets is greatly facilitated by inspection of peak intensities: the components forming a given multiplet are expected to exhibit (nearly) the same intensity. To illustrate this point, the resonance lines of spin system 2 were assumed to be more intense than those of spin system 1. Figure 3B shows that, in addition to chemical shift degeneracy in the conventionally sampled N_i - 1 dimensions, the central peaks of spin system 1 (as described in Figure 3A) and those of spin system 3 (peaks shown in lighter shade) overlap. In this case, the two spin systems exhibit degenerate chemical shifts in all but one dimension of an ND FT NMR spectrum. In (N,N_i)D GFT NMR, the bottom-up identification of multiplet components resolves and groups the signals of the two spin systems in the basic spectra, thus yielding the equivalent of the ND chemical shift correlation.

[0026]   Figures 4A-C illustrate magnetization transfer pathways of the following GFT NMR experiments: (5,2)D HACACONHN and (5,2)D HACA,CONHN (Figure 4A); (5,3)D HACACONHN and (5,3)D HACA,CONHN (Figure 4B); and (4,3)D CBCACONHN and (4,3)D CBCA,CONHN (Figure 4C). INEPT-type polarization transfers (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) are indicated by arrows, and Löhr's "en passant" frequency labeling module is indicated by a double arrow. The nuclei for which the chemical shift is detected in quadrature are shown in bold and are underlined. The nuclei with a grey background are simultaneously sampled in the GFT NMR dimension, and the chemical shifts of the boxed nuclei are used to establish sequential connectivities. In Figure 4B, the

chemical shifts of nitrogen spins shown in circles are measured is a separate dimension.

[0027]   Figures 5A-G illustrate magnetization transfer pathways generating the basic spectra of GFT NMR experiments: (i) (4,3)D HNNCACBCA (Figure 5A), (ii) (4,3)D HNN(CO)CACBCA and (4,3)D CBCACA(CO)NHN (Figure 5B), (iii) (5,3)D HBHACBCACA(CO)NHN (Figure 5C), (iv) (5,3)D HCC,CH-COSY (Figure 5D), (v) (5,3)D HBCBCGCDHD (Figure 5E), (vi) (4,2) HCCH-COSY (Figure 5F), and (vii) (5,2)D HCCCH-COSY (Figure 5G). In experiments (iv) and (vi), only magnetization transfer pathways corresponding to cross peaks in a 4D HCCH-COSY are shown. In experiment (vii), only the magnetization transfer pathway corresponding to cross peaks in a relayed 5D HCCCH-COSY is shown. INEPT-type polarization transfer are indicated by double arrows for "out-and-back" type experiments and single arrows for "out-and-stay" type experiments. The nucleus for which the chemical shift is detected in quadrature in all spectra constituting the GFT NMR experiment is underlined. The nuclei with grey background are simultaneously sampled in a single GFT NMR dimension, and the chemical shifts of the boxed nuclei are measured in the direct dimension. The chemical shifts of nitrogen spins (shown in circles) are measured in a separate dimension in experiments (i), (ii), and (iii), and the chemical shifts of $^{13}C^{\delta}$ and $^{13}C_i^{coupled}$ (shown in circles) are measured in a separate dimension in experiments (iv) and (v), respectively. The double headed arrows between $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ in experiments (i), (ii), and (iii) indicate that the chemical shifts of $^{13}C^{\alpha/\beta}$ [and $^{1}H^{\alpha/\beta}$ in (iii)] first evolve independently, prior to transferring to $^{13}C^{\alpha}$ for frequency labeling.

[0028]   Figure 6 illustrates the rf pulse sequence used to record the (5,2)D HACACONHN GFT NMR experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no rf phase is marked, the pulse is applied along x. The high power 90° pulse lengths were: 5.6 µs for ^{1}H and 15.3 µs for ^{13}C, and 39 µs for ^{15}N. Pulses on ^{13}C prior to $t_1(^{13}C)$ are applied at high power, and ^{13}C decoupling during $t_1(^{1}H)$ is achieved using a $(90_x\text{-}180_y\text{-}90_x)$ composite pulse (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety). Subsequently, the

$90°$ and $180°$ pulse lengths of $^{13}C^\alpha$ are adjusted to 51.6 µs and 46 µs, respectively, to minimize perturbation of the ^{13}CO spins (Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety). The width of the $90°$ pulses applied to ^{13}CO pulse is 51.6 µs and the corresponding $180°$ pulses are applied with same power. A SEDUCE (Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety) $180°$ pulse with a length 252 µs is used to decouple ^{13}CO during t_1. WALTZ16 (Ernst et al., <u>Principles of Nuclear Magnetic Resonance in One and Two Dimensions</u>, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) is employed to decouple ^1H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N (rf = 1.78 kHz) during acquisition. The SEDUCE sequence (rf = 1.0 kHz) is used for decoupling of $^{13}C^\alpha$ during the ^{15}N chemical shift evolution period. The ^1H rf carrier is placed at 4.78 ppm. The $^{13}C^\alpha$, $^{13}C'$ and ^{15}N rf carriers are set to 56.3 ppm, 174.3 and 119.3 ppm, respectively. The duration and strengths of the pulsed z- field gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (100 µs, 16 G/cm); G3 (1 ms, 24 G/cm); G4 (250 µs, 30 G/cm); G5 (1.5 ms, 20 G/cm); G6 (1.25 ms, 30 G/cm); G7 (500 µs, 8 G/cm); G8 (125 µs, 29.5 G/cm). All PFG pulses are of rectangular shape. The delays are: $\tau_1 = 1.6$ ms, $\tau_2 = 3.6$ ms, $\tau_3 = 4.4$ ms, $\tau_4 = \tau_5 = 24.8$ ms, $\tau_6 = 5.5$ ms, $\tau_7 = 4.6$ ms, $\tau_8 = 1$ ms. ^1H-frequency labeling is achieved in a semi constant-time fashion with t_1^a (0) = 1.79 ms, t_1^b (0) = 1 µs, t_1^c (0) = 1.791 ms, $\Delta t_1^a = 62.5$ µs, $\Delta t_1^b = 32.9$ µs, $\Delta t_1^c = -29.6$ µs. Hence, the fractional increase of the semi constant-time period with t_1 equals to $\lambda = 1 + \Delta t_1^c / \Delta t_1^a = 0.53$. Phase cycling: $\phi_0 = x$; $\phi_1 = x, -x$; $\phi_2 = x, x, -x, -x$; $\phi_3 = x$; $\phi_4 = 4x, 4(-x)$; ϕ_6(receiver) = 2(x,-x,-x,x). The sensitivity enhancement scheme of Kay et al., <u>J. Am. Chem. Soc.</u> 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G6 is inverted in concert with a $180°$ shift of ϕ_5. In case this enhancement scheme is not employed, quadrature detection is accomplished by altering the phase ϕ_0 according to States-TPPI (Cavanagh et al., <u>Protein NMR Spectroscopy</u>, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety). For the setting of the phases ϕ_0, ϕ_1, ϕ_2 and ϕ_3 see Example 4.

[0029]   Figures 7A-B depict the experimental scheme for the (5,2)D HACA,CONHN (Figure 7A) and (4,3)D CBCA,CONHN GFT NMR (Figure 7B) experiments. Rectangular 90° and 180° pulses are indicated by filled and open vertical bars or shaped pulses, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The high power 90° pulse lengths were: 5.8 μs for ^{1}H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. In Figure 7A, pulses on ^{13}C prior to $t_1(^{13}$C$)$ are applied at high power, and ^{13}C decoupling during $t_1(^1$H$)$ is achieved using a (90$_x$- 180$_y$-90$_x$) composite pulse (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety). Subsequently, the 90° and 180° pulse lengths of ^{13}Cα are adjusted to 51.5 μs and 46 μs, respectively, to minimize perturbation of the ^{13}CO spins. The width of the 90° pulses applied to ^{13}CO pulse is 52 μs and the corresponding 180° pulses are applied with same power. A SEDUCE (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) 180° pulse with a length 252 μs is used to decouple ^{13}CO during $t_1(^{13}$C$)$. WALTZ16 (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) is employed to decouple ^{1}H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N (rf = 1.78 kHz) during acquisition. The SEDUCE sequence (rf = 1.0 kHz) is used for decoupling of ^{13}Cα during the ^{15}N chemical shift evolution period. The ^{1}H rf carrier is placed at 4.78 ppm. The ^{13}Cα and ^{15}N rf carriers are set to 56.3 ppm and 119.3 ppm, respectively. All ^{13}C' pulses are laminar shifted (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) by 118 ppm relative to the ^{13}Cα carrier position. By setting the spectral width of the jointly sampled dimension to one half of 118 ppm, the apparent carrier position for sampling of ^{13}C' chemical shift (174.3 ppm) is folded on the position of the ^{13}Cα carrier position at 56.3 ppm. The duration and strengths of the pulsed z-field gradients (PFGs) are: G_1 (1 ms, 24 G/cm); G_2 (100 μs, 16 G/cm); G_3 (1 ms, 24 G/cm); G_5 (1.5 ms, 20 G/ cm); G_6 (1.25 ms, 30 G/cm); G_7 (500 μs, 8 G/cm); G_8 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. The delays are: τ_1 = 1.6 ms, τ_2 = 9.0 ms, τ_4 =11.0 ms, τ_5 = 22.0 ms, τ_6 = 5.5 ms, τ_7 = 4.6 ms, τ_8 = 1 ms. ^{1}H-frequency

labeling is achieved in a semi constant-time fashion with $t_1^a(0) = 1.7$ ms, $t_1^b(0) = 1$ µs, $t_1^c(0) = 1.701$ ms, $\Delta t_1^a = 60$ µs, $\Delta t_1^b = 35.4$ µs, $\Delta t_1^c = -24.6$ µs. Hence, the fractional increase of the semi constant-time period with t_1 equals to $\lambda = 1 + \Delta t_1^c / \Delta t_1^a = 0.58$. Phase cycling for artefact suppression: $\phi_0 = x$; $\phi_1 = x, -x$; $\phi_2 = x, x, -x, -x$; $\phi_3 = x$; $\phi_4 = 4x, 4(-x)$; $\phi_5 = x$; $\phi_6 = \phi_7 = x$; ϕ_8(receiver) $= 2(x,-x,-x,x)$. Phases ϕ_6 and ϕ_7 are shifted by 50° to compensate for non-resonance effects. GFT NMR super phase-cycling for recording the 8 basic spectra: $\phi_1 = x,y$; $\phi_2 = 2x,2y$; $\phi_3 = 4x,4y$ (the G-matrix required for time domain editing is shown in equation 15 in Example 5). For acquisition of central peaks derived from ^{13}C steady state magnetization, a second set of data sets with a 180° shift for ϕ_3 is collected and data are "pre-processed" as described (see equations 13 and 14 in Example 5). For second order central peak detection, the $^1H^\alpha$ and $^{13}C^\alpha$ chemical shift evolution periods are omitted and $\phi_1 = x,y$; $\phi_2 = x$; $\phi_3 = x$. Third order central peaks were detected in 2D $[^{15}N,^1H]$-HSQC (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety). (The G-matrices required for the central peak spectra are shown in equations 16-18 in Example 5). The sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G6 is inverted in concert with a 180° shift of ϕ_5. For implementation of (5,3)D HACA,CONHN, $t_1(^{15}N)$ is replaced by $t_2(^{15}N)$, and quadrature detection in t_1 is accomplished by altering the phase ϕ_1 according to States-TPPI. GFT NMR super phase cycle for the 4 basic spectra: $\phi_2 = x,y$; $\phi_3 = 2x,2y$ (the G-matrix required for time domain editing is shown in equation 16 of Example 5). First order central peaks are derived from ^{13}C magnetization and are obtained by acquiring a second set of data sets with a 180° shift for ϕ_3. For second order central peak detection, $t_1(^1H^\alpha)$ and $t_1(^{13}C^\alpha)$ are omitted. (The G-matrices required for time domain editing of the central peak spectra are shown in equations 17 and 18 of Example 5). In Figure 7B, pulses on ^{13}C prior to $t_1(^{13}C)$ are applied at high power. Subsequently, the 90° and 180° pulse lengths applied for $^{13}C^{\alpha/\beta}$ are adjusted to 47.5 µs and 42.5 µs, respectively, to minimize perturbation of ^{13}CO spins. The width of the 90° pulses applied to ^{13}CO pulse is 52 µs and the corresponding 180° pulses are applied with same power. SEDUCE 180° pulses of 200µs pulse length are used to decouple ^{13}CO during t_1 and

τ_4. WALTZ16 is employed to decouple ^{1}H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers, as well as to decouple ^{15}N (rf = 1.78 kHz) during acquisition. The SEDUCE sequence is used for decoupling of ^{13}C' during the ^{15}N chemical shift evolution period (rf = 1.0 kHz). The ^{1}H rf carrier is placed at 4.78 ppm. Initially, the ^{13}C and ^{15}N rf carriers are set to 41.3 ppm and 119.3 ppm, respectively. The ^{13}C' carrier position is folded from 174.3 to 41.3 ppm by setting the spectral width in ω_1 to one half of 133 ppm (= 174.3 ppm – 41.3 ppm). The ^{13}C carrier is set to 56.3 ppm during the τ_7 delay. The duration and strengths of the pulsed z-field gradients (PFGs) are: G_1 (1 ms, 24 G/ cm); G_2 (100 μs, 16 G/cm); G_3 (250 μs, 29.5 G/cm); G_4 (250 μs, 30 G/cm); G_5 (1.5 ms, 20 G/cm); G_6 (1.25 ms, 30 G/cm); G_7 (500 μs, 8 G/cm); G_8 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. The delays are: τ_0 = 1.7 ms, τ_1 = 800 μs, τ_2 = 2.8 ms, τ_3 = 3.3 ms, τ_4 = 6.6 ms, τ_6 = 8.8 ms, τ_7 = 24 ms, τ_8 = 5.5 ms, τ_9 = 4.6 ms, τ_{10} = 1.0 ms. Phase cycling for artefact suppression: ϕ_1 = x; ϕ_2 = 2(x), 2(-x); ϕ_3 = x; ϕ_4 = x, -x; ϕ_5 = ϕ_6 = ϕ_7 = ϕ_8 = x; ϕ_9(receiver) = x,-x,- x,x. Phases ϕ_6 and ϕ_7 are shifted by 120° to compensate for non-resonance effects. GFT NMR super phase-cycling for recording the two basic spectra: ϕ_2 = x,y (the G-matrix required for time domain editing is shown in equation 17 of Example 5). The sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G6 is inverted in concert with a 180° shift of ϕ_8. Quadrature detection in $t_1(^{13}C')$ is accomplished by altering the phase ϕ_6 according to States-TPPI (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety).

[0030]   Figure 8 depicts the experimental scheme for recording the (4,3)D CBCACONHN GFT NMR experiment. Rectangular 90° and 180° pulses are indicated by filled and open vertical bars or shaped pulses, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The high power 90° pulse lengths were: 5.8 μs for ^{1}H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. Pulses on ^{13}C prior to $t_1(^{13}C)$ are applied at high power. Subsequently, the 90° and 180° pulse lengths applied for $^{13}C\alpha/\beta$ are adjusted to 47.5 μs and 42.5 μs, respectively, to minimize perturbation of ^{13}CO spins. The

width of the 90° pulse applied on ^{13}CO pulse is 52 µs and the corresponding 180° pulses are applied with same power. A SEDUCE (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) 180° pulse with a length of 200 µs is used to decouple ^{13}CO during t_1 and

5   τ_4. The length of the spin-lock purge pulses SL_x and SL_y are 1.2 ms and 0.6 ms, respectively. WALTZ16 (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) is employed to decouple ^1H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N during acquisition (rf = 1.78 kHz) during

10   acquisition. The SEDUCE sequence is used for decoupling of $^{13}C^\alpha$ during ^{15}N evolution period (rf = 1.0 kHz). The ^1H rf carrier is placed at the water line at 4.78 ppm. Initially, the ^{13}C and ^{15}N rf carriers are set to 41.3 ppm and 119.3 ppm, respectively. The ^{13}C carrier is set to 56 ppm during the second $\tau_4/2$ delay. The $^{13}C'$ carrier position is set to 174.3 ppm. The duration and strengths of the pulsed z-field

15   gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (100 µs, 16 G/ cm); G3 (250 µs, 29.5 G/cm); G4 (250 µs, 30 G/cm); G5 (1.5 ms, 20 G/cm);G6 (1.25 ms, 30 G/ cm); G7 (500 µs, 8 G/cm); G8 (125 µs, 29.5 G/cm). All PFG pulses are of rectangular shape. The delays are: τ_1 = 800 µs, τ_2 = 3.1 ms, τ_3 = 3.6 ms, τ_4 = 7.2 ms, τ_5 = 4.4 ms, τ_6 = 24.8 ms, τ_7 = 24.8 ms, τ_8 = 5.5 ms, τ_9 = 4.6 ms, τ_{10} = 1.0 ms. Phase cycling for

20   artefact suppression: ϕ_1 = x; ϕ_2 = x,x,- x,-x; ϕ_3 = x, -x; ϕ_4 = x, -x; ϕ_5 = x; ϕ_6 = x, x, -x, -x; ϕ_7 = x; ϕ_8(receiver) = x, -x, -x, x. The sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G_6 is inverted in concert with a 180° shift of ϕ_7. Quadrature detection in $t_1(^{13}C')$ is accomplished by altering the

25   phase ϕ_4 according to States-TPPI. GFT NMR super phase-cycle for acquisition of the two basic spectra: ϕ_2 = x,y (the G-matrix required for time domain editing is shown in equation 17 of Example 5). For first order central peak detection an HNNCO pulse scheme (Cavanagh et al., Protein NMR spectroscopy, Wiley, New York (1996), which is hereby incorporated by reference in its entirety) is employed.

30   [0031]   Figure 9 depicts the experimental scheme for the (4,3)D HNNCACBCA experiment. Rectangular 90° and 180° pulses are indicated by thin

and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The high-power 90° pulse lengths were: 6.0 μs for ^1H, 15.0 μs for ^{13}C and 42 μs for ^{15}N. The 90° and 180° pulse lengths applied on $^{13}C^{\alpha/\beta}$ are adjusted to 40 μs and 36 μs,

5   respectively, to minimize perturbation of ^{13}CO spins. One lobe sinc pulses of duration 65 μs were applied on ^{13}CO with null at $^{13}C^{\alpha}$ to decouple ^{13}CO from $^{13}C^{\alpha}$ spins during t_1 and from ^{15}N spins during t_2. The selective 90° ^1H pulse used to flip back the water magnetization is applied for 1.8 ms duration before the first 90° pulse on $^{13}C^{\alpha}$. WALTZ16 is employed to decouple ^1H (rf field strength = 9.2 kHz) during the

10   heteronuclear magnetization transfers as well as to decouple of ^{15}N (rf = 1.78 kHz) during acquisition. The ^1H rf carrier is placed at the position of the solvent line at 4.78 ppm. The $^{13}C^{\alpha}$ and ^{15}N carriers are set to 43.0 ppm and 120.9 ppm, respectively. The ^{13}C carrier is switched to 56 ppm during the second t_1 delay. The duration and strengths of the pulsed z- field gradients (PFGs) are: G1 (1.0 ms, 24 G/cm); G2 (100

15   μs, 16 G/cm); G3 (500 μs, 29.5 G/cm); G5 (100 μs, 16 G/cm); G4 (1.5 ms, 20 G/cm); G6(1.5 ms, 20 G/cm); G7 (1.25 ms, 30 G/cm); G8 (500 μs, 8 G/cm); G9 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an rf pulse. The delays have the following values: τ_1 = 4.6 ms, τ_2 = 5.4 ms, τ_3 = 24 ms, τ_4 = 24 ms, τ_5 = 4.8 ms, τ_c =

20   7.0 ms. Phase cycling: ϕ_1 = x, -x; ϕ_2 = y; ϕ_3 = x,x, -x, -x; ϕ_4 =x, ϕ_5 = 4(x), 4(-x); ϕ_6 =x; ϕ_7(receiver) = x, -x, -x, x. The sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G7 is inverted in concert with a 180° shift of ϕ_6. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases

25   ϕ_3 and ϕ_4, respectively, according to States-TPPI. GFT-NMR super phase-cycling for recording the 2 basic spectra are: ϕ_1 = x,y; ϕ_2 = y,x.

[0032]   Figure 10 depicts the experimental scheme for the (4,3)D HNN(CO)CACBCA experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses.

30   Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The high-power 90° pulse lengths were: 6.0 μs for ^1H, 15.0 μs for ^{13}C and 42 μs for ^{15}N.

The 90° and 180° pulse lengths applied on $^{13}C^{\alpha/\beta}$ are adjusted to 40 μs and 36 μs, respectively, to minimize perturbation of ^{13}CO spins. One lobe sinc pulses of duration 65 μs and with null at $^{13}C^{\alpha}$ were applied on ^{13}CO to decouple from $^{13}C^{\alpha}$ spins during t_1 and from ^{15}N spins during t_2. The 90° pulse lengths for the one lobe sinc pulse on ^{13}CO was 71 μs. The selective 90° ^1H pulse used to flip back the water magnetization is applied for 1.8 ms duration before the first 90° pulse on $^{13}C^{\alpha}$. WALTZ16 is employed to decouple ^1H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple of ^{15}N (rf = 1.78 kHz) during acquisition. The ^1H rf carrier is placed at the position of the solvent line at 4.78 ppm. The $^{13}C^{\alpha}$ and ^{15}N carriers are set to 43 ppm and 120.9 ppm, respectively. The ^{13}C carrier is switched to 56 ppm during the second t_1 delay. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1.0 ms, 24 G/cm); G2 (100 μs, 16 G/cm); G3 (1.0 ms, 29.5 G/ cm); G4 (1.5 ms, 20 G/cm); G5 (100 μs, 16 G/cm); G6(1.5 ms, 20 G/cm); G7 (1.25 ms, 30 G/cm); G8 (500 μs, 8 G/cm); G9 (125 μs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an rf pulse. The delays have the following values: τ_1 = 4.4 ms, τ_2 = 5.4 ms, τ_3 = 24 ms, τ_4 = 24 ms, τ_5 = 4.8 ms, τ_a = 4.6 ms, τ_b = 6.8 ms, τ_c = 6.9 ms. Phase cycling: ϕ_1 = x, -x; ϕ_2 = y; ϕ_3 = x,x, -x, -x; ϕ_4 =x, ϕ_5 = 4(x), 4(-x); ϕ_6 =x; ϕ_7(receiver) = x, -x, -x, x. The sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G7 is inverted in concert with a 180° shift of ϕ_6. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases ϕ_3 and ϕ_4, respectively, according to States-TPPI. GFT-NMR super phase-cycle for recording the 2 basic spectra are: ϕ_1 = x,y; ϕ_2 = y,x.

[0033]   Figure 11 depicts the experimental scheme for the (4,3)D CBCACA(CO)NHN experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The high-power 90° pulse lengths were: 5.9 μs for ^1H, 15.4 μs for ^{13}C, and 38 μs for ^{15}N. Pulses on ^{13}C prior to $t_1(^{13}C)$ are applied at high power, and ^{13}C decoupling during $t_1(^1H)$ is achieved using a (90$_x$-180$_y$-90$_x$) composite pulse. Subsequently, the 90° and

180° pulse lengths applied for $^{13}C^{\alpha/\beta}$ are adjusted to 47.5 μs and 42.5 μs, respectively, to minimize perturbation of ^{13}CO spins. The width of the 90° pulse applied on ^{13}CO pulse is 52 μs and the corresponding 180° pulses are applied with same power. A SEDUCE 180° pulse with a length of 200 μs is used to decouple ^{13}CO during t_1 and

5   τ_4. The length of the spin-lock purge pulses SL_x and SL_y are 1.2 ms and 0.6 ms, respectively. WALTZ16 is employed to decouple ^1H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N during acquisition (rf = 1.78 kHz) during acquisition. The SEDUCE sequence is used for decoupling of $^{13}C^{\alpha}$ during ^{15}N evolution period (rf = 1.0 kHz). The ^1H rf carrier is

10   placed at 4.78 ppm. Initially, the ^{13}C and ^{15}N r. f. carriers are set to 43 ppm and 120.9 ppm, respectively. The ^{13}C carrier is set to 56 ppm before the first $\tau_4/2$ delay period. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (100 μs, 16 G/cm); G3 (250 μs, 29.5 G/cm); G4 (250 μs, 30 G/cm); G5 (1.5 ms, 20 G/cm);G6 (1.25 ms, 30 G/cm); G7 (500 μs, 8 G/cm); G8 (125 μs, 29.5

15   G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an rf pulse. The delays are: τ_1 = 600 μs, τ_2 = 3.1 ms, τ_3 = 3.35 ms, τ_4 = 6.8 ms, τ_5 = 4.4 ms, τ_6 = 24.6 ms, τ_7 = 24.6 ms, τ_8 = 5.5 ms, τ_9 = 4.6 ms, τ_{10} = 1.0 ms. Phase cycling: ϕ_1 = x; ϕ_2 = x,x,- x,-x; ϕ_3 = x, -x; ϕ_4 = x, -x; ϕ_5 = x; ϕ_6 = x, x, -x, -x; ϕ_7 = x; ϕ_8 = x; ϕ_9(receiver) = x, -x, -x, x. The

20   sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G6 is inverted in concert with a 180° shift of ϕ_7. GFT-NMR super phase-cycling for recording the 2 basic spectra are: ϕ_2 =x,y. Quadrature detection in $t_1(^{13}C)$ and $t_2(^{15}N)$ is accomplished by altering the phases ϕ_8 and ϕ_5, respectively, according

25   to States-TPPI.

[0034]   Figure 12 depicts the experimental scheme for the (5,3)D HBHACBCACA(CO)NHN experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along

30   x. The scaling factor κ for ^1H chemical shift evolution during t_1 is set to 1.0. The high-power 90° pulse lengths were: 5.9 μs for ^1H, 15.4 μs for ^{13}C, and 38 μs for ^{15}N.

Pulses on ^{13}C prior to $t_1(^{13}$C) are applied at high power, and ^{13}C decoupling during $t_1(^1$H) is achieved using a $(90_x-180_y-90_x)$ composite pulse. Subsequently, the 90° and 180° pulse lengths applied for ^{13}C$^{\alpha/\beta}$ are adjusted to 47.5 µs and 42.5 µs, respectively, to minimize perturbation of ^{13}CO spins. The width of the 90° pulse applied on ^{13}CO

5   pulse is 52 µs and the corresponding 180° pulses are applied with same power. A SEDUCE 180° pulse with a length of 200 µs is used to decouple ^{13}CO during t_1 and τ_4. The length of the spin-lock purge pulses SL$_x$ and SL$_y$ are 1.2 ms and 0.6 ms, respectively. WALTZ16 is employed to decouple ^{1}H (rf field strength = 9.2 kHz) during the heteronuclear magnetization transfers as well as to decouple ^{15}N during

10   acquisition (rf = 1.78 kHz) during acquisition. The SEDUCE sequence is used for decoupling of ^{13}C$^{\alpha}$ during ^{15}N evolution period (rf = 1.0 kHz). The ^{1}H rf carrier is placed at -1 ppm before the start of the semi constant time ^{1}H chemical shift evolution period, and then switched to the water line at 4.78 ppm after the second 90° ^{1}H pulse. Initially, the ^{13}C and ^{15}N r. f. carriers are set to 43 ppm and 120.9 ppm, respectively.

15   The ^{13}C carrier is set to 56 ppm during the second $\tau_4/2$ delay. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (1 ms, 24 G/cm); G2 (100 µs, 16 G/cm); G3 (250 µs, 29.5 G/cm); G4 (250 µs, 30 G/ cm); G5 (1.5 ms, 20 G/cm);G6 (1.25 ms, 30 G/cm); G7 (500 µs, 8 G/cm); G8 (125 µs, 29.5 G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 µs duration is inserted

20   between a PFG pulse and an rf pulse. The delays are: τ_1 = 600 µs, τ_2 = 3.1 ms, τ_3 = 3.35 ms, τ_4 = 6.8 ms, τ_5 = 4.4 ms, τ_6 = 24.6 ms, τ_7 = 24.6 ms, τ_8 = 5.5 ms, τ_9 = 4.6 ms, τ_{10} = 1.0 ms. ^{1}H-frequency labeling, at a ^{1}H resonance frequency of 600 MHz is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) = 1 µs, t_1^c (0) = 1.701 ms, Δt_1^a = 33.3 µs, Δt_1^b = 19.3 µs, Δt_1^c = -14 µs. Hence, the fractional increase

25   of the semi constant-time period with t_1 equals to λ = 1 + Δt_1^c / Δt_1^a = 0.58. Phase cycling: ϕ_1 = x; ϕ_2 = x,x,-x,-x; ϕ_3 = x, -x; ϕ_4 = x, -x; ϕ_5 = x; ϕ_6 = x, x, -x, -x; ϕ_7 = x; ϕ_8 = x; ϕ_9(receiver) = x, -x, -x, x. The sensitivity enhancement scheme of Kay et al., J. Am. Chem. Soc. 114:10663–10665 (1992), which is hereby incorporated by reference in its entirety, is employed, i.e., the sign of G$_6$ is inverted in concert with a 180° shift

30   of ϕ_7. Quadrature detection in $t_1(^{13}$C) and $t_2(^{15}$N) is accomplished by altering the phases ϕ_8 and ϕ_5, respectively, according to States-TPPI. GFT-NMR super phase-

cycling for recording the 4 basic spectra are: ϕ_1 = x,y; ϕ_2 = x,y. For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 shifted by 180°, is collected.

[0035] Figure 13 depicts the experimental scheme for the (5,3)D HCC,CH-COSY experiment. Rectangular 90° and 180° pulses are indicated by thin and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The scaling factor κ for ^1H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.8 μs for ^1H and 15.4 μs for ^{13}C, and 38 μs for ^{15}N. The lengths of the ^1H spin-lock purge pulses are: first SL_x, 2.8 ms; second SL_x, 1.7 ms; SL_y: 4.9 ms. SEDUCE is used for decoupling of ^{13}CO during t_1 and t_2 (rf field strength = 1 kHz). WURST is used for decoupling of ^{13}C during acquisition. The ^1H carrier is placed at the position of the solvent line at 0 ppm before the start of the first semi constant time ^1H evolution period, and then switched to the water line at 4.78 ppm after the second 90° ^1H pulse. The ^{13}C and ^{15}N rf carriers are set to 43 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (500 μs, 6 G/cm); G2 (500 μs, 11 G/cm); G3 (100 μs, 12 G/cm); G4 (100 μs, 12.5 G/cm); G5 (4.0 ms, 22 G/cm);G6 (500 μs, 5 G/cm); G7 (3.0 ms, 22 G/cm); G8 (400 μs, 6 G/cm). All gradients are applied along z-axis and are of rectangular shape. All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an rf pulse. The delays are: τ_1 = 1.6 ms, τ_2 = 750 μs, τ_3 = 2.65 ms, τ_4 = 3.4 ms, τ_5 = 6.8 ms, τ_6 = 1.6 ms, τ_7 = 2.4 ms, τ_a = 350 μs, τ_b = 1.65 ms and τ_c = 2.4 ms. Phase cycling: ϕ_1 = x; ϕ_2 = x, -x; ϕ_3 = x, -x; ϕ_4 = x; ϕ_5 = y; ϕ_6(receiver) = x, -x. Quadrature detection in $t_1(^{13}C/^1H)$ and $t_2(^{13}C)$ is accomplished by altering the phases ϕ_4 and ϕ_5, respectively, according to States-TPPI. Water suppression is accomplished by coherence pathway rejection using spin-lock purge pulses and pulsed field z-gradients. GFT-NMR super phase-cycle for recording the 4 basic spectra are: ϕ_1=x,y; ϕ_2=x,y. For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 shifted by 180° is collected.

[0036] Figure 14 depicts the experimental scheme for the (5,3)D HBCBCGCDHD experiment. Rectangular 90° and 180° pulses are indicated by thin

and thick vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The scaling factor κ for ^1H chemical shift evolution during t_1 is set to 1.0. The high power 90° pulse lengths were: 5.8 μs for ^1H and 15.4 μs for ^{13}C. The first 180° pulse on ^{13}C prior to $t_1(^{13}C)$ is applied at high power. Subsequently, the 90° pulse lengths of $^{13}C^\beta$ is adjusted to 66 μs. The 180° $^{13}C^\beta$ and $^{13}C^{aro}$ pulses are of gaussian-3 shape and 375 μs duration. WALTZ16 is used for decoupling of ^1H (rf field strength = 4.5 kHz) during the magnetization transfer from $^{13}C^\alpha$ to $^{13}C^{aro}$, and GARP is employed to decouple $^{13}C^{aro}$ (rf = 2.5 kHz) during acquisition. The ^1H rf carrier is placed at 4.78 ppm. The ^{13}C rf carrier is set to 38 ppm during $\omega_1(^{13}C^\beta)$ and then switched to 135 ppm before the first 90° pulse on $^{13}C^{aro}$ (pulse labeled with ϕ_4). The ^{13}C rf carrier is switched back to 125 ppm before the second 90° pulse on $^{13}C^{aro}$. The duration and strengths of the pulsed z- field gradients (PFGs) are: G1 (500 μs, 2 G/cm); G2 (1 ms, 22 G/cm); G3 (2 ms, 10 G/cm); G4 (500 μs, 4 G/cm); G5 (1 ms, -14 G/cm); G6 (500 μs, -2G/cm). All PFG pulses are of rectangular shape. A recovery delay of at least 100 μs duration is inserted between a PFG pulse and an rf pulse. The delays are: τ_1 = 1.8 ms, τ_2 = 8.8 ms, τ_3 = 71 μs, τ_4 = 4.3 ms, τ_5 = 2.1 ms, τ_6 = 710 μs, τ_8 = 1.4 ms, τ_7 = 2.5 ms. ^1H-frequency labeling, at a ^1H resonance frequency of 600 MHz is achieved in a semi constant-time fashion with t_1^a (0) = 1.7 ms, t_1^b (0) = 1 μs, t_1^c (0) = 1.701 ms, Δt_1^a = 33.3 μs, Δt_1^b = 19.3 μs, Δt_1^c = -14 μs. Hence, the fractional increase of the semi constant-time period with t_1 equals to $\lambda = 1 + \Delta t_1^c / \Delta t_1^a = 0.58$. Phase cycling: ϕ_1 = x; ϕ_2 = x; ϕ_3 = x, y, -x, -y; ϕ_4 = 4(x), 4(-x); ϕ_5 = x; ϕ_6 (receiver) = x, -x, x, -x, -x, x, -x, x. Quadrature detection in $t_1(^{13}C^\delta)$ and $t_2(^{13}C^\gamma)$ is accomplished by altering the phases ϕ_4 and ϕ_5, respectively, according to States-TPPI. Water suppression is accomplished by presaturation of the water line during the relaxation delay and coherence pathway rejection using spin-lock purge pulses and pulsed field z-gradients. GFT-NMR super phase-cycling for recording the 4 basic spectra are: ϕ_1=x,y; ϕ_2=x,y. For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 shifted by 180° is collected.

[0037]   Figure 15 depicts the experimental scheme for the (4,2)D HCCH-COSY experiment. Rectangular 90° and 180° pulses are indicated by thin and thick

vertical bars, respectively, and phases are indicated above the pulses. Where no radio-frequency (rf) phase is marked, the pulse is applied along x. The high power $90°$ pulse lengths were: 5.8 µs for ^1H and 15.4 µs for ^{13}C, and 38 µs for ^{15}N. The lengths of the ^1H spin-lock purge pulses are: first SL_x, 2.8 ms; second SL_x, 1.7 ms; SL_y: 4.9 ms. SEDUCE is used for decoupling of ^{13}CO during t_1 and t_2 (rf field strength = 1 kHz). WURST is used for decoupling of ^{13}C during acquisition. The ^1H carrier is placed at 4.78 ppm. The ^{13}C and ^{15}N rf carriers are set to 43 ppm and 120.9 ppm, respectively. The duration and strengths of the pulsed z-field gradients (PFGs) are: G1 (500 µs, 6 G/cm); G2 (500 µs, 11 G/cm); G3 (100 µs, 12 G/cm); G4 (100 µs, 12.5 G/cm); G5 (4 ms, 22 G/cm);G6 (500 µs, 5 G/cm); G7 (3ms, 30 G/cm); G8 (400 µs, 6 G/cm). All gradients are applied along z-axis and are of rectangular shape. All PFG pulses are of rectangular shape. A recovery delay of at least 100 µs duration is inserted between a PFG pulse and an rf pulse. The delays are: τ_1 = 1.6 ms, τ_2 = 750 µs, τ_3 = 2.65 ms, τ_4 = 3.4 ms, τ_5 = 6.8 ms, τ_6 = 0.7 ms, τ_7 = 3.2 ms. Phase cycling: ϕ_1 = x; ϕ_2 = x, -x; ϕ_3 = x, -x; ϕ_4 = x; ϕ_5 = y; ϕ_6(receiver) = x, -x. Quadrature detection in $t_1(^{13}C)$ is accomplished by altering the phases ϕ_4 according to States-TPPI. Water suppression is accomplished by coherence pathway rejection using spin-lock purge pulses and pulsed field z-gradients. GFT-NMR super phase-cycle for recording the 4 basic spectra are: ϕ_1 = x,y; ϕ_2 = x,y. For acquisition of central peaks derived from ^{13}C steady state magnetization, a second data set with ϕ_1 shifted by $180°$ is collected.

[0038]   Figures 16A-B show the $\omega_1[(^{15}N;^{13}C',^{13}C^\alpha,^1H^\alpha), \omega_2(^1H^N)]$-, $[\omega_1(^{15}N;^{13}C',^{13}C^\alpha), \omega_2(^1H^N)]$-, $[\omega_1(^{15}N;^{13}C'), \omega_2(^1H^N)]$-, and $[\omega_1(^{15}N), \omega_2(^1H^N)]$-strips taken from the (5,2)D <u>HACACONHN</u> GFT NMR experiment (see Figure 17). The signals were detected on the amide proton chemical shift of Ser 20. Figure 16A shows spectra $A1$-$A15$ containing the chemical shift multiplets. Figure 16B shows spectra $B1$-$B15$ containing the individual edited chemical shift multiplet components. Note that when compared with Figure 2 the order of the chemical shift multiplets appears to have changed. However, this is because $\omega_1(^1H^\alpha) < 0$ ppm (i.e., upfield relative to the carrier position) for Ser 20, and $\omega_1(^{13}C')$, $\omega_1(^{13}C^\alpha)$ and $\omega_1(^{15}N) > 0$ ppm (i.e, downfield relative to the respective carrier position). For simplicity, Figure 2 was designed with the assumption that all resonances are located downfield to the

respective carrier positions. The signals located at higher field in $A15$ and $B15$ arise from a side chain moiety and have thus no corresponding peaks in the other spectra (see also Figure 17D). To facilitate the comparison of Figures 16A and 16B, the positions of multiplet components are indicated with thin lines.

[0039]   Figures 17A-E show the 15 2D planes constituting the (5,2)D HACACONHN GFT NMR experiment ($K = 3$) recorded for the 8.6 kDa protein ubiquitin. The linear combination of chemical shifts detected in a given plane is indicated. Figure 17A shows the basic spectra $B1$ to $B8$. Figure 17B shows the first order central peak spectra $B9$ to $B12$. Figure 17C shows the second order central peak spectra $B13$ and $B14$. Figure 17D shows the third order central peak spectrum $B15$. Signals arising from side chain moieties are in dashed boxes. Figure 17E shows cross sections taken along $\omega_1(^{15}N;^{13}C',^{13}C^{\alpha},^{1}H^{\alpha})$ at the peak of Ser 20 in $B1$ (at the top), along $\omega_1(^{15}N;^{13}C',^{13}C^{\alpha})$ in $B9$ (second from top), along $\omega_1(^{15}N;^{13}C')$ in $B13$ (third from top), and along $\omega_1(^{15}N)$ in $B15$ (at the bottom). The sections are indicated in green in the corresponding panel. Comparison of sections from $B1$ and $B9$ shows that signals do not broaden with increasing K (Figure 18), while the smaller line widths observed in spectra $B13$ to $B15$ result from longer t_{max} values (see Example 4). The 15 signals detected on the backbone amide proton of Ile 36 are circled. Doublets are observed in $B1$-$B8$ since Gly 35 exhibits non-degenerate $^{1}H^{\alpha}$ chemical shifts, yielding the correlation of six shifts: $\delta(^{1}H^{\alpha 2}) = 4.135 \pm 0.006$ ppm, $\delta(^{1}H^{\alpha 1}) = 3.929 \pm 0.006$ ppm, $\delta(^{13}C^{\alpha}) = 46.10 \pm 0.019$ ppm, $\delta(^{13}C') = 173.911 \pm 0.017$ ppm for Gly 35, and $\delta(^{15}N) = 120.295 \pm 0.043$ ppm and $\delta(^{1}H^{N}) = 6.174 \pm 0.005$ ppm for Ile 36 (Table 2). The standard deviations of the indirectly detected chemical shifts were estimated from a Monte Carlo simulation (see description of Figure 19). In accordance, the $\omega_2(^{1}H^{N})$ line width of the directly detected amid proton (20 Hz) was identified with $\pm 3\sigma$ (99.5% confidence interval) for locating the peak positions. Notably, phase sensitive editing of the chemical shift multiplets yields increasing peak dispersion (and thus resolution) in each of the constituent spectra compared to 2D $[^{15}N,^{1}H]$-HSQC (panel $B15$). Nearly the same number of peaks is detected in each of 15 spectra, while the spectral width increases from $SW_1(^{15}N) = 1,440$ Hz in $B15$ to $SW_1(^{15}N/^{13}C'/^{13}C^{\alpha}/^{1}H^{\alpha}) = 8,000$ Hz in $B1...B8$.

[0040]   Figures 18A-C compare line widths and digital resolution of peaks detected in GFT and FT NMR. Figure 18A shows (5,2)D HACACONHN GFT NMR: cross sections taken along $\omega_1({}^{15}N;{}^{13}C',{}^{13}C^\alpha,{}^1H^\alpha)$ at the peak of Ser 20 in spectrum $B1$ (at the top), along $\omega_1({}^{15}N;{}^{13}C',{}^{13}C^\alpha)$ in spectrum $B9$ (second from top), along $\omega_1({}^{15}N;{}^{13}C')$ in spectrum $B13$ (third from top), and along $\omega_1({}^{15}N)$ in spectrum $B15$ (at the bottom). The same t_{max} value was chosen for all spectra in order to demonstrate that resonances do not broaden when increasing K from 0 to 3. Figure 18B shows HACACONHN FT NMR: $\omega_1({}^1H^\alpha)$, $\omega_1({}^{13}C^\alpha)$, $\omega_1({}^{13}C')$, and $\omega_1({}^{15}N)$ cross sections taken from 2D [ω_1, $\omega_2({}^1H^N)$]- planes obtained with the HACACONHN rf pulse scheme which were (i) recorded with the same t_{max} values and spectral widths, and (ii) were processed as (5,2)D HACACONHN. Comparison of Figure 18A and Figure 18B shows that the linewidth registered in the GFT NMR experiment equals the linewidth in the FT NMR experiment. Figure 18C shows the same cross sections as in Figure 18B are shown except that the planes were recorded and processed as a conventional 5D NMR spectrum would be [same maximal evolution times as in the basic spectra, $10(t_1)*11(t_2)*22(t_3)*13(t_4)*512(t_5)$ complex points with spectral widths of $SW_1({}^{15}N) = 1,440$ Hz, $SW_2({}^{13}C') = 1,500$ Hz, $SW_3({}^{13}C^\alpha) = 3,260$ Hz, and $SW_4({}^1H^\alpha) = 1,800$ Hz and linear prediction to $20(t_1)*22(t_2)*32(t_3)*26(t_4)*512(t_5)$ complex points]. This would yield a frequency domain data set of $32(\omega_1)*32(\omega_2)*32(\omega_3)*32(\omega_4)*512(\omega_5)$ real points of 2.1 GByte size as compared to 16.8 MByte for (5,2)D HACACONHN. Comparison with Figure 18B and Figure 18C makes the relatively poor resolution obtainable in 5D FT NMR apparent. Note that linear prediction and zero filling to $96(\omega_1)*96(\omega_2)*256(\omega_3)*128(\omega_4)*512(\omega_5)$ real points, which would be the closest match to the digital resolution obtained in (5,2)D HACACONHN, would result in an unrealistically large data size of 618 GByte.

[0041]   Figure 19 illustrates Monte-Carlo simulations performed to assess the increased precision of chemical shift measurements in (5,2)D HACACONHN GFT NMR. Standard deviations for the chemical shift measurements are plotted *versus* the number of spectra selected from the 15 2D spectra constituting this experiment (Figure 17) in order to calculate the chemical shifts. $\sigma({}^1H^\alpha)$, $\sigma({}^{13}C^\alpha)$, $\sigma({}^{13}C')$ and $\sigma({}^{15}N)$ represent the deviations for $\Omega_3({}^1H^\alpha)$, $\Omega_2({}^{13}C^\alpha)$, $\Omega_1({}^{13}C')$ and $\Omega_0({}^{15}N)$

measurements, respectively. The following conservative statistical model is adopted. Line widths at half height, $\Delta v_{1/2}$, were measured along ω_1 in (i) $B1$-$B12$ (basic spectra and first order central peaks) providing $\Delta v_{1/2}$(basic) = $\Delta v_{1/2}$(first) = 60.1 Hz, (ii) $B13$ and $B14$ (second order central peaks) providing $\Delta v_{1/2}$(second) = 38.2 Hz and (iii) $B15$

5   (third order central peaks) providing $\Delta v_{1/2}$(3rd) = 28.1 Hz [Figure 17E; these values are close to those expected from the t_{max} values obtained after linear prediction]. It is then assumed that the error for the identification of peak positions is associated with a Gaussian distribution, and that the Lorenzian line width, $\Delta v_{1/2}$, represents $\pm 3\sigma$ (99.5% confidence interval), i.e., $\Delta v_{1/2} = 6\sigma$. $\Delta v_{1/2}$(basic) is equal to the line widths in

10   the indirect dimensions of conventional FT NMR spectra recorded with the same maximal evolution time (Figures 17E and 18). Hence, σ (basic) likewise represents the standard deviation obtained in FT NMR. Correspondingly are σ (second) = $\Delta v_{1/2}$(second)/6 and σ (third) = $\Delta v_{1/2}$(third)/6 the standard deviations for peak position identification in $B13$ and $B14$, and $B15$. The deviations $\sigma(^1H^\alpha)$, $\sigma(^{13}C^\alpha)$,

15   $\sigma(^{13}C')$ and $\sigma(^{15}N)$ were obtained from Monte Carlo simulations of error propagation for which the following systems of equations were considered: (i) a minimal number of four out of the eight basic spectra ($B1$, $B4$, $B6$, $B7$; Figure 20) (ii) $B1$-$B8$, (iii) $B1$-$B12$, (iv) $B1$-$B14$, or (v) $B1$-$B15$. Peak positions were randomly varied 10,000 times according to Gaussian distributions characterized by σ(basic), σ(second) and σ(third).

20   Subsequently, the systems of equations were solved using a least-squares fitting routine, and the deviations among the 10,000 solutions yielded σ ($^1H^\alpha$), $\sigma(^{13}C^\alpha)$, $\sigma(^{13}C')$ and $\sigma(^{15}N)$. Note that $\sigma(^1H^\alpha)$ is not further reduced when central peaks are involved since those do not encode $\Omega(^1H^\alpha)$. Similarly, $\sigma(^{13}C^\alpha)$ and $\sigma(^{13}C')$ are not further reduced when second and third order central peaks are considered for

25   calculation of chemical shifts. Notably, the standard deviations (labeled with an asterisk) obtained with four spectra critically depend on the particular selection (Figure 20). The highest precision is obtained when choosing either $B1$, $B4$, $B6$ and $B7$, or $B2$, $B3$, $B5$ and $B8$ (Figures 20 and 17). The simulations are in neat agreement with calculations using the Gaussian law of error propagation (see Figure 20).

30   **[0042]**   Figures 20A-E show the results of Monte-Carlo simulations for the case that only four out of eight basic spectra of (5,2)D HACACONHN (Figure 17A)

are selected to calculate the chemical shifts. The standard deviations for the chemical shift measurements are plotted *versus* the number assigned to a particular combination. Figures 20A-D show $\sigma(^{15}N)$, $\sigma(^{13}C')$, $\sigma(^{13}C^{\alpha})$ and $\sigma(^{1}H^{\alpha})$, respectively, which represent the standard deviations for the measurement of the chemical shifts $\Omega_0(^{15}N)$, $\Omega_1(^{13}C')$, $\Omega_2(^{13}C^{\alpha})$ and $\Omega_3(^{1}H^{\alpha})$, respectively. Figure 20E illustrates the assignment of numbers to the selections of four out of the 64 possible combinations $\{\binom{8}{4} - 6 = (8\cdot7\cdot6\cdot5)/(4\cdot3\cdot2\cdot1) - 6 = 64\}$. The six combinations which are subtracted from the binomial coefficient $\binom{8}{4}$ correspond to the cases where one of the three chemical shifts Ω_1, Ω_2 or Ω_3 is added to or subtracted from Ω_0 in all of the four selected spectra (i.e., no splitting is present among the four selected spectra which encodes the respective chemical shift). The spectra selected for a particular combination number are indicated as dots. The statistical model used for the Monte Carlo simulations is the same as described in the legend of Figure 19.

[0043]   Figures 21A-B show the composite plot of $[\omega_1,\omega_2]$-strips taken from (5,2)D HACA,CONHN (Figure 21A) and HACACONHN data (Figure 21B) collected for the 8.6 kDa protein ubiquitin with a total measurement time of 10.5 hours. The 2D data were acquired with 58(t_1) : 512(t_2) complex points and $t_{1max}(^{15}N; ^{13}C', ^{13}C^{\alpha}, ^{1}H^{\alpha}) = 6.5$ ms and $t_{2max}(^{1}H^{N}) = 73.2$ ms. In Figure 21A, the strips were taken from basic spectra ($B1$ to $B8$), first order central peak spectra ($B9$ to $B12$), second order central peak spectra ($B13$ and $B14$) and third order central peak spectra ($B15$) and are centered about the amide proton chemical shift of Glu 64. The position of the backbone ^{15}N chemical shift of Glu 64 is indicated by a dashed horizontal line, and the type of linear combination of chemical shifts detected for a given strip along ω_1 is indicated at the top of the strip: $B1$ $[\Omega_0 + \Omega_1 + \Omega_2 + \Omega_3]$; $B2$ $[\Omega_0 - \Omega_1 + \Omega_2 + \Omega_3]$; $B3$ $[\Omega_0 + \Omega_1 - \Omega_2 + \Omega_3]$; $B4$ $[\Omega_0 - \Omega_1 - \Omega_2 + \Omega_3]$; $B5$ $[\Omega_0 + \Omega_1 + \Omega_2 - \Omega_3]$; $B6$ $[\Omega_0 - \Omega_1 + \Omega_2 - \Omega_3]$; $B7$ $[\Omega_0 + \Omega_1 - \Omega_2 - \Omega_3]$; $B8$ $[\Omega_0 - \Omega_1 - \Omega_2 - \Omega_3]$; $B9$ $[\Omega_0 + \Omega_1 + \Omega_2]$; $B10$ $[\Omega_0 - \Omega_1 + \Omega_2]$; $B11$ $[\Omega_0 + \Omega_1 - \Omega_2]$; $B12$ $[\Omega_0 - \Omega_1 - \Omega_2]$; $B13$ $[\Omega_0 + \Omega_1]$; $B14$ $[\Omega_0 - \Omega_1]$; $B15$ $[\Omega_0]$. In Figure 21B, the corresponding strips are centered about the amide proton chemical shift of Ser 65. The variation of the 15 peaks relative to the ^{15}N chemical shift of Ser 65 (indicated by a dashed horizontal line) matches the variation about the ^{15}N chemical shift of Glu 64 in Figure 21A. This allows one to establish the

sequential connectivity between Glu 64 and Ser 65 based on the measurement of three chemical shifts, i.e., $\Omega(^{13}C'), \Omega(^{13}C^{\alpha})$ and $\Omega(^{1}H^{\alpha})$. The shifts are obtained with high precision (Table 3) since the errors are reduced by the following factors when compared with FT NMR. For $\Omega(^{15}N)$: $\sqrt{15} = 3.9$; $\Omega(^{13}C')$: $\sqrt{14} = 3.7$; $\Omega(^{13}C^{\alpha})$:

5    $\sqrt{12} = 3.5$; $\Omega(^{1}H^{\alpha})$: $\sqrt{8} = 2.8$. ^{1}H and ^{13}C chemical shifts are in ppm relative to 2,2-dimethyl- 2-silapentane-5-sulfonate sodium salt (DSS).

[0044]   Figure 22 shows the composite plot of $[\omega_1, \omega_3]$-strips taken from (5,3)D HACACONHN (strips labeled with 'a') and (5,3)D HACA,CONHN data (strips labeled with 'b') collected for the 14 kDa NESG consortium target protein TT212

10   with a total measurement time of 60 hours. The 3D data were acquired with $56(t_1)$ $\cdot 24(t_2) \cdot 512(t_3)$ complex points and $t_{1max}(^{13}C'; {}^{13}C^{\alpha}, {}^{1}H^{\alpha}) = 6.2$ ms, $t_{2max}(^{15}N) = 16.4$ ms and $t_{3max}(^{1}H^{N}) = 73.2$ ms. The first, second and third pair of strips in each block has been taken, respectively, at the ^{15}N chemical shift of Ala 24, Ile 25 and Glu 26 along $\omega_2(^{15}N)$. The strips are centered about the corresponding amide proton shifts

15   detected along $\omega_3(^{1}H^{N})$. The ^{15}N shifts are given at the bottom of each pair of strips, which were taken from basic spectra ($B1$ to $B4$), the first order central peak spectra ($B5$ and $B6$) and the second order central peak spectra ($B7$). The type of linear combination of chemical shifts detected along ω_1 is indicated at the top of the strips: $B1 [\Omega_0 + \Omega_1 + \Omega_2]$; $B2 [\Omega_0 - \Omega_1 + \Omega_2]$; $B3 [\Omega_0 + \Omega_1 - \Omega_2]$; $B4 [\Omega_0 - \Omega_1 - \Omega_2]$; $B5 [\Omega_0$

20    $+ \Omega_1]$; $B6 [\Omega_0 - \Omega_1]$; $B7 [\Omega_0]$. Sequential connectivities are indicated by horizontal lines and are established based on the measurement of three chemical shifts, i.e., $\Omega(^{13}C'), \Omega(^{13}C^{\alpha})$, and $\Omega(^{1}H^{\alpha})$. The chemical shifts were obtained with high precision (Table 4), since the errors are reduced by the following factors when compared with FT NMR. For $\Omega(^{13}C')$: $\sqrt{7} = 2.6$; $\Omega(^{13}C^{\alpha})$: $\sqrt{6} = 2.4$; $\Omega(^{1}H^{\alpha})$: $\sqrt{4} = 2$. ^{1}H and

25   ^{13}C chemical shifts are in ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS).

[0045]   Figure 23 shows the composite plot of $[\omega_1, \omega_3]$-strips taken from (4,3)D CBCACONHN (strips labeled with 'a') and (4,3)D CBCA,CONHN data (strips labeled with 'b') collected for the 8.6 kDa protein ubiquitin with a total measurement

30   time of 11.2 hours. The 3D data were acquired with $60(t_1) \cdot 24(t_2) \cdot 512(t_3)$ complex points and $t_{1max}(^{13}C'; {}^{13}C^{\alpha/\beta}) = 5.9$ ms, $t_{2max}(^{15}N) = 17.2$ ms and $t_{3max}(^{1}H^{N}) = 73.2$ ms.

The first, second, and third pair of strips in each block has been taken, respectively, at the ^{15}N chemical shift of Glu 64, Ser 65, and Thr 66 along $\omega_2(^{15}$N). The strips are centered about the corresponding amide proton shifts detected along $\omega_3(^1$H^N). The ^{15}N shifts are given at the bottom of each pair of strips, which were taken from basic spectra ($B1$ and $B2$) and the first order central peak spectra ($B3$). The type of linear combination of chemical shifts detected along ω_1 is indicated at the top of the strips: $B1$ [$\Omega_0 + \Omega_1$]; $B2$ [$\Omega_0 - \Omega$]; $B3$ [Ω_0]. Sequential connectivities are indicated by horizontal lines and are established based on the measurement of three chemical shifts, i.e., $\Omega(^{13}$C'$), \Omega(^{13}$C$^\alpha)$ and $\Omega(^{13}$C$^\beta)$. [Since the ^{13}C$^{\alpha/\beta}$ carrier was set in between the ^{13}C$^\alpha$ and ^{13}C$^\beta$ chemical shift ranges (Figure 7), one has that peaks at $\omega_2(^{13}$C' + ^{13}C$^\alpha)$ and $\omega_2(^{13}$C' + ^{13}C$^\beta)$ in B1 appear in a "reversed order" when compared with B2, which exhibits peaks at $\omega_2(^{13}$C' - ^{13}C$^\alpha)$ and $\omega_2(^{13}$C' - ^{13}C$^\beta)$.] The chemical shifts were obtained with high precision (Table 5) since the errors are reduced by the following factors when compared with FT NMR. For $\Omega(^{13}$C'): $\sqrt{3} = 1.7$; $\Omega(^{13}$C$^\alpha)$: $\sqrt{2} = 1.4$; $\Omega(^{13}$C$^\beta)$: $\sqrt{2} = 1.4$. ^{1}H and ^{13}C chemical shifts are in ppm relative to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS).

[0046]   Figure 24 shows a composite plot of [ω_1,ω_3]-strips taken from (5,3)D HACACONHN (strips labeled with 'a') and (5,3)D HACA,CONHN data (strips labeled with 'b') collected for ubiquitin with a total measurement time of 20.8 hours. The 3D data were acquired with 56(t_1) · 24(t_2) · 512(t_3) complex points and $t_{1max}(^{13}$C'; ^{13}C$^\alpha$, ^{1}H$^\alpha) = 6.2$ ms, $t_{2max}(^{15}$N$) = 17.2$ ms and $t_{3max}(^1$H$^N) = 73.2$ ms. The first, second, and third pair of strips in each block has been taken, respectively, at the ^{15}N chemical shift of Lys 63, Glu 64, and Ser 65 along $\omega_2(^{15}$N). The strips are centered about the corresponding amide proton shifts detected along $\omega_3(^1$H^N). The ^{15}N shifts are given at the bottom of each pair of strips, which were taken from basic spectra ($B1$ to $B4$), the first order central peak spectra ($B5$ and $B6$) and the second order central peak spectra ($B7$). The type of linear combination of chemical shifts detected along ω_1 is indicated at the top of the strips: $B1$ [$\Omega_0 + \Omega_1 + \Omega_2$]; $B2$ [$\Omega_0 - \Omega_1 + \Omega_2$]; $B3$ [$\Omega_0 + \Omega_1 - \Omega_2$]; $B4$ [$\Omega_0 - \Omega_1 - \Omega_2$]; $B5$ [$\Omega_0 + \Omega_1$]; $B6$ [$\Omega_0 - \Omega_1$]; $B7$ [Ω_0]. Sequential connectivities are indicated by horizontal lines and are established based on the measurement of three chemical shifts, i.e., $\Omega(^{13}$C'$), \Omega(^{13}$C$^\alpha)$ and $\Omega(^1$H$^\alpha)$. The chemical shifts were obtained

with high precision (Table 6), since the errors are reduced by the following factors when compared with FT NMR. For $\Omega(^{13}C')$: ; $\Omega(^{13}C^\alpha)$: ; $\Omega(^1H^\alpha)$: . ^1H and ^{13}C chemical shifts are in ppm relative to 2,2- dimethyl-2-silapentane-5-sulfonate sodium salt (DSS).

[0047]   Figure 25 shows the composite plot of $[\omega_1(^{13}C^\alpha; {}^{13}C^{\alpha/\beta}), \omega_3(^1H^N)]$ strips taken from the basic spectra of (a) (4,3)D HNNCACBCA (B1a, B2a) and (b) (4,3)D HNN(CO)CACBCA (B1b, B2b). The $[\omega_1(^{13}C^\alpha), \omega_3(^1H^N)]$ strips taken from 3D HNNCA (B3a) and 4D HNN(CO)CA (B3b) spectra represent the first order central peaks for (4,3)D HNNCACBCA and (4,3)D HNN(CO)CACBCA, respectively. As an example, strips corresponding to $\omega_2(^{15}N)$ and $\omega_3(^1H^N)$ chemical shifts for the residue Glu 73 of the 16 kDa protein ER75 are shown. Dashed lines connecting peaks establish sequential connectivities. Peaks labeled 1 to 9 in the figure correspond to the following linear combination of chemical shifts ($i \equiv$ Glu 73; $i-1 \equiv$ Ala 71):

1: $\Omega_0(^{13}C^\alpha_{i-1}) + \Omega_1(^{13}C^\beta_{i-1})$;

2: $\Omega_0(^{13}C^\alpha_i) + \Omega_1(^{13}C^\beta_i)$

3: $\Omega_0(^{13}C^\alpha_i) + \Omega_1(^{13}C^\alpha_i)$, $\Omega_0(^{13}C^\alpha_{i-1}) + \Omega_1(^{13}C^\alpha_{i-1})$

4: $\Omega_0(^{13}C^\alpha_i) - \Omega_1(^{13}C^\alpha_i)$

5: $\Omega_0(^{13}C^\alpha_{i-1}) - \Omega_1(^{13}C^\alpha_{i-1})$

6: $\Omega_0(^{13}C^\alpha_i) - \Omega_1(^{13}C^\beta_i)$

7: $\Omega_0(^{13}C^\alpha_{i-1}) - \Omega_1(^{13}C^\beta_{i-1})$

8: $\Omega_0(^{13}C^\alpha_i)$

9: $\Omega_0(^{13}C^\alpha_{i-1})$

[0048]   Figure 26 shows the composite plot of $[\omega_1(^{13}C^\alpha; {}^{13}C^{\alpha/\beta}), \omega_3(^1H^N)]$ strips taken from the basic spectra of (a) (4,3)D CBCACA(CO)NHN (B1a and B2a) and (b) (4,3)D HNNCACBCA (B1b and B2b) illustrating how sequential resonance assignments along the polypeptide chain are obtained. As an example, the sequential walk for residues Val 27 to Ile 30 of the 7 kDa protein GR2 is shown. For simplicity,

only the sequential connectivities inferred from the basic spectra are shown. The observed peak patterns are as described in Figure 25.

[0049]   Figure 27 shows the composite plot of $[\omega_1(^{13}C^\alpha; {}^{13}C^{\alpha/\beta}, {}^1H^{\alpha/\beta}),$ $\omega_3(^1H^N)]$ strips taken from the basic and first order central peak spectra of (5,3)D HBHACBCACA(CO)NHN (B1a, B2a, B3a and B4a). Note that $[\omega_1(^{13}C^\alpha; {}^{13}C^{\alpha/\beta}),$ $\omega_3(^1H^N)]$ strips taken from the basic spectra of (4,3)D CBCACA(CO)NHN (B5b and B6b) show the same peak patterns as those observed in the first order central peak spectra of (5,3)D HBHACBCACA(CO)NHN (B5a and B6a). As an example, strips corresponding to $\omega_2(^{15}N)$ and $\omega_3(^1H^N)$ chemical shifts for Ile 30 of GR2 are shown. Peaks labeled 1 to 12 in the figure correspond to the following linear combination of chemical shifts for residue Ile 29:

1: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$

2: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\beta) + \Omega_2(^1H^\beta)$

3: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$

4: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\beta) - \Omega_2(^1H^\beta)$

5: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\beta) - \Omega_2(^1H^\beta)$

6: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$

7: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\beta) + \Omega_2(^1H^\beta)$

8: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$

9: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\beta)$

10: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\alpha)$

11: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\alpha)$

12: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\beta)$

[0050]   Figure 28 shows the composite plot of $[\omega_1(^{13}C; {}^{13}C, {}^1H), \omega_3(^1H)]$ strips taken from the basic (B1-B4) and first order central peak (B5 and B6) spectra of (5,3)D HCC,CH-COSY. The $[\omega_1(^{13}C), \omega_3(^1H)]$ strips taken from 3D (H)C,CH-COSY (B7) represents the second order central peak spectra of (5,3)D HCC,CH-COSY. As an example, strips corresponding to $\omega_2(^{13}C^\alpha)$ and $\omega_3(^1H^\alpha)$ chemical shifts for residue

Ile 30 of GR2 are shown. Peaks shown in rectangular boxes correspond to cross peaks in a conventional 4D HCCH-COSY. Peaks labeled 1 to 13 correspond to the following linear combination of chemical shifts:

5

Corresponding peak type in 4D HCCH-COSY (peaks 1-8) and 3D CCH-COSY (peaks 9-12)

1: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$ "Diagonal peak"

2: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\beta) + \Omega_2(^1H^\beta)$ "Cross peak"

10   3: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$ "Diagonal peak"

4: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\beta) - \Omega_2(^1H^\beta)$ "Cross peak"

5: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\beta) + \Omega_2(^1H^\beta)$ "Cross peak"

6: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$ "Diagonal peak"

7: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\beta) - \Omega_2(^1H^\beta)$ "Cross peak"

15   8: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$ "Diagonal peak"

9: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\alpha)$ "Diagonal peak"

10: $\Omega_0(^{13}C^\alpha) + \Omega_1(^{13}C^\beta)$ "Cross peak"

11: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\beta)$ "Cross peak"

12: $\Omega_0(^{13}C^\alpha) - \Omega_1(^{13}C^\alpha)$ "Diagonal peak"

20   13: $\Omega_0(^{13}C^\alpha)$

[0051]   Figure 29 shows the composite plot of $[\omega_1(^{13}C^\delta; {}^{13}C^\beta, {}^1H^\beta), \omega_3(^1H^\delta)]$ strips taken from the basic (B1-B4) and first order central peak (B5 and B6) spectra of (5,3)D HBCBCGCDHD illustrating how resonance assignments for aromatic side-chain spins are obtained. The $[\omega_1(^{13}C^\delta; {}^{13}C^\beta), \omega_3(^1H^\delta)]$ strips taken from 3D $[^{13}C^\delta,$
25   $^{13}C^\gamma, H^\delta]$-COSY represent the second order central peak spectra of (5,3)D HBCBCGCDHD. As an example, strips corresponding to $\omega_2(^{13}C^\gamma)$ and $\omega_3(^1H^\delta)$ chemical shifts for His 68 of Ubiquitin are shown. Peaks labeled 1 to 7 correspond to the following linear combination of chemical shifts:

1: $\Omega_0(^{13}C^{\delta 2}) - \Omega_1(^{13}C^\beta) - \Omega_2(^1H^\beta)$

2: $\Omega_0(^{13}C^{\delta 2}) - \Omega_1(^{13}C^{\beta}) + \Omega_2(^1H^{\beta})$

3: $\Omega_0(^{13}C^{\delta 2}) + \Omega_1(^{13}C^{\beta}) + \Omega_2(^1H^{\beta})$

4: $\Omega_0(^{13}C^{\delta 2}) + \Omega_1(^{13}C^{\beta}) - \Omega_2(^1H^{\beta})$

5: $\Omega_0(^{13}C^{\delta 2}) - \Omega_1(^{13}C^{\beta})$

6: $\Omega_0(^{13}C^{\delta 2}) + \Omega_1(^{13}C^{\beta})$

7: $\Omega_0(^{13}C^{\delta 2})$

[0052]   Figure 30 shows the composite plot of $[\omega_1(^{13}C; {}^{13}C, {}^1H), \omega_2(^1H)]$ strips taken from the basic (B1-B4) and first order central peak (B5 and B6) spectra of (4,2)D HCCH-COSY spectra illustrating how resonance assignments for aromatic side-chain spins are obtained. The $[\omega_1(^{13}C), \omega_2(^1H)]$ strip taken from 2D $[^{13}C{-}^1H]$ HSQC (B7) represents the second order central peak spectra for (4,2)D HCCH-COSY. As an example, strips corresponding to $\omega_2(^1H^{\varepsilon})$ chemical shift for residue Tyr 59 of the 8.6 kDa protein Ubiquitin are shown. Peaks shown in rectangular boxes correspond to cross peaks in the conventional 4D HCCH-COSY. Peaks labeled 1 to 15 correspond to the following linear combination of chemical shifts:

	Corresponding peak type in 4D HCCH-COSY (peaks 1-8) and 3D CCH-COSY (peaks 9-14)
1: $\Omega_0(^{13}C^{\varepsilon}) - \Omega_1(^{13}C^{\delta}) - \Omega_2(^1H^{\delta})$	"Cross peak"
2: $\Omega_0(^{13}C^{\varepsilon}) - \Omega_1(^{13}C^{\varepsilon}) - \Omega_2(^1H^{\varepsilon})$	"Diagonal peak"
3: $\Omega_0(^{13}C^{\varepsilon}) - \Omega_1(^{13}C^{\delta}) + \Omega_2(^1H^{\delta})$	"Cross peak"
4: $\Omega_0(^{13}C^{\varepsilon}) - \Omega_1(^{13}C^{\varepsilon}) + \Omega_2(^1H^{\varepsilon})$	"Diagonal peak"
5: $\Omega_0(^{13}C^{\varepsilon}) + \Omega_1(^{13}C^{\varepsilon}) + \Omega_2(^1H^{\varepsilon})$	"Diagonal peak"
6: $\Omega_0(^{13}C^{\varepsilon}) + \Omega_1(^{13}C^{\delta}) + \Omega_2(^1H^{\delta})$	"Cross peak"
7: $\Omega_0(^{13}C^{\varepsilon}) + \Omega_1(^{13}C^{\varepsilon}) - \Omega_2(^1H^{\varepsilon})$	"Diagonal peak"
8: $\Omega_0(^{13}C^{\varepsilon}) + \Omega_1(^{13}C^{\delta}) - \Omega_2(^1H^{\delta})$	"Cross peak"
9: $\Omega_0(^{13}C^{\varepsilon}) - \Omega_1(^{13}C^{\varsigma})$	"Cross peak"
10: $\Omega_0(^{13}C^{\varepsilon}) - \Omega_1(^{13}C^{\delta})$	"Cross peak"

11: $\Omega_0(^{13}C^\varepsilon) - \Omega_1(^{13}C^\varepsilon)$ "Diagonal peak"

12: $\Omega_0(^{13}C^\varepsilon) + \Omega_1(^{13}C^\varepsilon)$ "Diagonal peak"

13: $\Omega_0(^{13}C^\varepsilon) + \Omega_1(^{13}C^\delta)$ "Cross peak"

14: $\Omega_0(^{13}C^\varepsilon) + \Omega_1(^{13}C^\varsigma)$ "Cross peak"

15: $\Omega_0(^{13}C^\varepsilon)$

DETAILED DESCRIPTION OF THE INVENTION

[0053]   The present invention provides an NMR data acquisition scheme which is based on the phase sensitive joint sampling of the indirect dimensions spanning a subspace of a conventional NMR experiment. This allows one to very rapidly obtain high dimensional NMR spectral information. Since the phase-sensitive joint sampling yields subspectra containing "chemical shift multiplets", alternative data processing is required for editing the components of the multiplets. The subspectra are linearly combined using a so-called "G- matrix" and subsequently Fourier transformed. The chemical shifts are multiply encoded in the resonance lines constituting the shift multiplets. This corresponds to performing statistically independent multiple measurements, and the chemical shifts can thus be obtained with high precision. To indicate that a combined G-matrix and FT is employed, the new approach is named "GFT NMR spectroscopy".

[0054]   In GFT NMR spectroscopy, the chemical shift evolution periods spanning a given multidimensional subspace of an FT NMR experiment are "jointly" sampled (Figure 1). Thereby, the dimensionality N of an FT NMR spectrum can be adjusted to a given target dimensionality, N_t, by combined sampling of $K + 1$ chemical shifts $\Omega_0, \Omega_1, \ldots \Omega_K$ encoded in $K + 1$ indirect dimensions of the ND FT NMR experiment ($K = N - N_t$). Assuming that Ω_0 is detected in quadrature (Ernst et al., <u>Principles of Nuclear Magnetic Resonance in One and Two Dimensions</u>, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) and that the setting of the phases ϕ_j of the radiofrequency pulses exciting the spins of dimension j ($j = 1\ldots K$) ensures cosine modulation, the transfer amplitude (Ernst et al., <u>Principles of Nuclear Magnetic Resonance in One and Two Dimensions</u>, Clarendon, Oxford

(1987), which is hereby incorporated by reference in its entirety) of the N_tD experiment is proportional to $e^{i\Omega_0 t} \cdot \prod_{j=1}^{K} \cos(\Omega_j t)$. The resulting peak centered around Ω_0 contains 2^K components and is designated a "chemical shift multiplet" (Figure 2).

[0055]   A shift of ϕ_j by $90°$ yields a $\sin(\Omega_j t)$ instead of a $\cos(\Omega_j t)$ modulation, (Ernst et al., <u>Principles of Nuclear Magnetic Resonance in One and Two Dimensions</u>, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety), and $2^K N_t$D spectra are recorded if all phases ϕ_k are systematically varied between $0°$ and $90°$ (Figure 1). In turn, a linear combination of these 2^K spectra allows for the editing of the chemical shift multiplet components (Figure 2). For brevity,

$$c_j = \cos(\Omega_j \cdot t), \quad s_j = \sin(\Omega_j \cdot t), \text{ and } e^{i\Omega_j t} = e^{i_j} \text{ are defined, so that}$$

$$e^{i_j} = c_j + i \cdot s_j = [1\, i] \cdot \begin{bmatrix} c_j \\ s_j \end{bmatrix}. \text{ With } K = 1, \text{ one obtains for the time evolution of the two}$$

shift multiplet components encoding sum and difference of Ω_0 and Ω_1.

$$\begin{bmatrix} e^{i_1} \\ e^{-i_1} \end{bmatrix} \otimes e^{i_0} = e^{i_0} = \begin{bmatrix} e^{i_1} \cdot e^{i_1} \\ e^{-i_1} \cdot e^{i_0} \end{bmatrix} = \begin{bmatrix} [1\,i] \otimes [1\,i] \\ [1-i] \otimes [1\,i] \end{bmatrix} \cdot \left[\begin{bmatrix} c_1 \\ s_1 \end{bmatrix} \otimes \begin{bmatrix} c_0 \\ s_0 \end{bmatrix} \right] = \begin{bmatrix} 1\,i \\ 1\,-i \end{bmatrix} \otimes [1\,i] \cdot \left[\begin{bmatrix} c_1 \\ s_1 \end{bmatrix} \otimes \begin{bmatrix} c_0 \\ s_0 \end{bmatrix} \right]$$

Accordingly, one obtains with $K = 2$ for three chemical shifts Ω_0, Ω_1, and Ω_2:

$$\begin{bmatrix} e^{i_2} \\ e^{-i_2} \end{bmatrix} \otimes \begin{bmatrix} e^{i_1} \\ e^{-i_1} \end{bmatrix} \otimes e^{i_0} = \begin{bmatrix} 1\,i \\ 1\,-i \end{bmatrix} \otimes \begin{bmatrix} 1\,i \\ 1\,-i \end{bmatrix} \otimes [1\,i] \cdot \left[\begin{bmatrix} c_2 \\ s_2 \end{bmatrix} \otimes \begin{bmatrix} c_1 \\ s_1 \end{bmatrix} \otimes \begin{bmatrix} c_0 \\ s_0 \end{bmatrix} \right],$$

and, in general, for $K + 1$ chemical shifts $\Omega_0, \Omega_1, \ldots \Omega_K$:

$$\begin{bmatrix} e^{i_K} \\ e^{-i_K} \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} e^{i_1} \\ e^{-i_1} \end{bmatrix} \otimes e^{i_0} = \begin{bmatrix} 1\,i \\ 1\,-i \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} 1\,i \\ 1\,-i \end{bmatrix} \otimes [1\,i] \cdot \left[\begin{bmatrix} c_K \\ s_K \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} c_1 \\ s_1 \end{bmatrix} \otimes \begin{bmatrix} c_0 \\ s_0 \end{bmatrix} \right]$$

The 2^K dimensional complex vector on the left side of the equation is proportional to the vector $\hat{T}_c(K)$ comprising the desired edited spectra with the individual components of the chemical shift multiplets, that is,

$$\hat{T}_c(K) \sim \begin{bmatrix} e^{i_K} \\ e^{-i_K} \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} e^{i_1} \\ e^{-i_1} \end{bmatrix} \otimes e^{i_0}$$

The 2^{K+1} dimensional real vector of the 2^{K+1} trigonometric modulations on the right side of the equation is proportional to the vector containing the spectra with the

chemical shift multiplets in the real, S_{jr}, and imaginary parts, S_{ji}, of the 2^K N_tD spectra $(j = 1 \ldots 2^K)$. Hence, with $\hat{S}(K) = [S_{1r}\,S_{1i}\,S_{2r}\,S_{2i}\ldots S_{2^K r}\,S_{2^K i}]^T$,

$$\hat{S}(K) \sim \begin{bmatrix} c_K \\ s_K \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} c_1 \\ s_1 \end{bmatrix} \otimes \begin{bmatrix} c_0 \\ s_0 \end{bmatrix}$$

For the $2^K \times 2^{K+1}$ complex G-matrix, which transforms $\hat{S}(K)$ into $\hat{T}(K)$ according to the following equation (1):

$$\hat{T}_c(K) = \hat{G}_c(K) \cdot \hat{S}(K) \tag{1}$$

one then obtains

$$G_c(K) = \left[\begin{bmatrix} 1 & i \\ 1 & -i \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} 1 & i \\ 1 & -i \end{bmatrix} \otimes [1\ i] \right]$$

Alternatively, the multiplet components may be edited in the frequency domain (Figure 2). The spectra of $\hat{S}(K)$ are Fourier transformed and a zero-order phase correction of $n \cdot 90^o$ is applied, depending on the number n of chemical shift sine modulations (see Example 1). The resulting real parts contain purely absorptive chemical shift multiplets and form the 2^K dimensional real vector $\hat{A}(K)$. Their linear combination yields the edited spectra contained in the 2^K dimensional real vector according to the following equation (2):

$$\hat{B}(K) = \hat{F}(K) \cdot \hat{A}(K) \tag{2}$$

Hence, $\hat{B}(K)$ represents spectra which contain the edited 2^K individual multiplet components at $\Omega_0 \pm \Omega_1 \pm \ldots \Omega_K$ encoding the desired $K + 1$ chemical shifts. $\hat{F}(K)$ can be readily obtained from $\hat{F}(K-1)$ by tensor product formation using the relation $\hat{F}(K) = \hat{F}(K-1) \otimes \hat{F}(1)$, with

$$F(1) = \begin{bmatrix} 1 & 1 \\ 1 & -1 \end{bmatrix}$$

(for details and the relation between F and the G-matrix see Example 1).

[0056] The 2^K spectra of $\hat{T}_c(K)$ and $\hat{B}(K)$ are designated "basic spectra". Additional information is required to unambiguously derive all shift correlations of the parent ND experiment (which resolves degeneracy in up to $N-1$ dimensions) if two multiplets exhibit degenerate chemical shifts in all of the "conventionally" sampled N_t -1 dimensions. The acquisition of peaks defining the centers of the chemical shift splittings ("central peaks") at the frequencies $\Omega_0 + \Omega_1 \pm ... \pm \Omega_{K-1}$,

$\Omega_0 \pm \Omega_1 \pm ... \pm \Omega_{K-2}, ..., \Omega_0 \pm \Omega_1$, and Ω_0 is then needed for identifying the components forming a given multiplet (Figure 3A). Such "central peak acquisition" has been introduced in the framework of the reduced-dimensionality NMR approach (Szyperski et al., Proc. Natl. Acad. Sci. USA, 99:8009-8014 (2002); Szyperski et al. J. Biomol. NMR. 3:127–132 (1993); Szyperski et al., J. Am. Chem. Soc. 115:9307-9308 (1993); Szyperski et al., J. Magn. Reson. B 105:188-191 (1994); Brutscher et al., J. Magn. Reson. B 105:77-82 (1994); Szyperski et al., J. Magn. Reson. B 108:197-203 (1995); Brutscher et al., J. Magn. Reson. B 109:238-242 (1995); Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Bracken et al., J. Biomol. NMR 9:94-100 (1997); Szyperski et al., J. Biomol NMR, 11:387-405 (1998); Astrof et al., J. Magn. Reson. 152:303-307 (2001); Xia et al., J. Biomol. NMR 24:41-40 (2002), which are hereby incorporated by reference in their entirety). The shift correlations of the ND spectrum can be obtained by "bottom-up" identification of the shift multiplets. This procedure essentially groups the peaks of the basic spectra into sets each belonging to one multiplet (Figure 3). Because the basic peaks of two spin systems can be grouped even if central peaks overlap (Figure 3B), this approach ensures that all correlations of the ND experiment are retained. GFT NMR (Figure 1) thus requires one to record a total of $p = \sum_{k=0}^{K} 2^k = 2^{K+1} - 1$ N_tD spectra, including 2^K basic spectra and 2^K-1 central peak spectra. The p data sets constitute an "$(N,N_t)D$ GFT NMR experiment", and central peaks arising from omission of m chemical shifts are denoted to be of m-th order. For practical purposes, it is important to note that all components of a given multiplet have quite similar intensities since they are generated by multiple sine or cosine modulation of the transfer amplitude. Usually this does not hold for two peaks belonging to two different spin systems (Figure 3A), because the nuclear spin relaxation times determining the peak intensities vary from spin system to spin

system. Hence, inspection of peak intensities greatly facilitates the grouping of the peaks.

[0057]   The joint sampling of several indirect dimensions reduces the minimal measurement time, T_m, of an (N,N_i)D GFT NMR experiment when compared with the parent ND FT experiment. The $K + 1$ dimensions of an FT NMR spectrum exhibiting the spectral widths SW_0, SW_1,....,SW_K are sampled with n_0, n_1,...n_K complex points and yield maximal evolution times of $t_{0,max}$, $t_{1,max}$,... $t_{K,max}$. In the (N,N_i)D GFT NMR experiment, the same maximal evolution times of the parent ND experiment can be realized by appropriate scaling of increments. (Szyperski et al. J. Biomol. NMR. 3:127–132 (1993); Szyperski et al., J. Magn. Reson. B 105:188-191 (1994), which are hereby incorporated by reference in their entirety). The acquisition of both cosine and sine modulated spectra for all jointly sampled chemical shifts (equation 1) corresponds to their phase-sensitive acquisition (Brutscher et al., J. Magn. Reson. B 109:238-242 (1995), which is hereby incorporated by reference in its entirety) and allows one to place the rf carrier positions in the center of the spectral ranges. Hence,

the spectral width required for combined sampling is given by $SW = \sum_{j=0}^{K} \kappa_j \cdot SW_j$,

where κ_j represents the factor to scale (Szyperski et al. J. Am. Chem. Soc. 115:9307–9308 (1993); Szyperski et al., J. Magn. Reson. B 105:188-191 (1994), which are hereby incorporated by reference in their entirety) the sampling increments of the jth dimension to adjust maximal evolution times. If the same maximal evolution time is chosen for all dimensions and assuming, for simplicity, that delayed acquisition starts at $1/SW_j$, $n = \sum_{j=0}^{K} n_j$ complex points are required to sample the resulting single

dimension [if acquisition starts at $t = 0$, one obtains that $n = \left(\sum_{j=0}^{K} n_j \right) - K$]. The ratio ε of the minimal measurement time of an FT NMR experiment, T_m(FT), and the corresponding GFT NMR experiment, T_m(GFT), is then given by the number of FIDs that are required to sample the $K+1$ FT NMR dimensions divided by p times the number of FIDs required to sample the resulting single dimension:

$$\varepsilon = \frac{T_m(FT)}{T_m(GFT)} = (2^K /(2^{K+1} - 1)) \cdot \left(\prod_{j=0}^{K} n_j \right) / \left(\sum_{j=0}^{K} n_j \right) \tag{3}$$

This ratio scales with the product of the number of points over the corresponding sum and, thus, predicts large reductions in T_m (see Table 1 in Example 3; different ways to implement central peak acquisition as well as the impact of a particular implementation on ε are described in Examples 2 and 3). (The GFT NMR scheme can be generalized by its M-fold application. Since this would involve M different G-matrices, such an experiment could be designated a G^MFT NMR experiment. For example, two groups of dimensions can be identified with each group being combined to a single dimension. First an (N,N')D experiment is devised in which dimensions 1,2....i are jointly sampled. Subsequently, the dimensionality of this experiment is to reduced to an (N,N_i) experiment by jointly sampling dimensions $i+1$, $i+2$,....$K+2$. For M projection steps, each invoking different sets of dimensions combined to a single one, the total reduction in minimal measurement time is then given by $\varepsilon^{tot} = \prod_{j=1}^{M} \varepsilon_j$,

where ε_j is the reduction due to the j-th projection (equation 3)). The S/N of each of the 2^K components in the basic spectra is reduced by $\left(1/\sqrt{2}\right)^K$ compared to the single peak in FT NMR. This is because each chemical shift splitting reduces the S/N by a factor of 2 relative to the FT NMR spectrum, while a factor of $\sqrt{2}$ is gained, because frequency discrimination is not associated with a FT (see Figure 2: both cosine and sine modulated parts contribute equally to the signal intensity in the edited spectra) (The S/N ratio of FT NMR can be recovered by symmetrization about central peaks as described for reduced-dimensionality NMR (Szyperski et al., J. Magn. Reson. B 108:197-203 (1995), which is hereby incorporated by reference in its entirety) using the "bottom up" strategy employed for identification of shift multiplets (Figure 3). Note that a reduced sensitivity is not relevant in the sampling limited regime.)

[0058]   GFT NMR spectroscopy combines (i) multiple phase sensitive RD NMR, (ii) multiple 'bottom-up' central peak detection, and (iii) (time domain) editing of the components of the chemical shift multiplets. The resulting formalism embodies a flexible, generally applicable NMR data acquisition scheme. Provided that m = K + 1 chemical shift evolution periods of an ND experiments are jointly sampled in a single indirect "GFT dimension", p = 2^m - 1 different $(N-K)$D spectra represent the GFT NMR experiment containing the information of the parent ND experiment.

Hence, such a set of p spectra is named an $(N,N-K)$D GFT NMR experiment.

[0059]   Thus, the present invention relates to a method of conducting a $(N,N-K)$ dimensional (D) G-matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiment, where N is the dimensionality of an N-dimensional (ND) Fourier transformation (FT) NMR experiment and K is the desired reduction in dimensionality relative to N. The method involves providing a sample and applying radiofrequency pulses for the ND FT NMR experiment to the sample. Then, m indirect chemical shift evolution periods of the ND FT NMR experiment are selected, where m equals $K+1$, and the m indirect chemical shift evolution periods are jointly sampled. Next, NMR signals detected in a direct dimension are independently cosine and sine modulated to generate $(N-K)$D basic NMR spectra containing frequency domain signals with 2^K chemical shift multiplet components, thereby enabling phase-sensitive sampling of all jointly sampled m indirect chemical shift evolution periods. Finally, the $(N-K)$ D basic NMR spectra are transformed into $(N-K)$ D phase-sensitively edited basic NMR spectra, where the 2^K chemical shift multiplet components of the $(N-K)$ D basic NMR spectra are edited to yield $(N-K)$ D phase-sensitively edited basic NMR spectra having individual chemical shift multiplet components.

[0060]   As described earlier, the $(N-K)$ D basic NMR spectra can be transformed into $(N-K)$ D phase-sensitively edited basic NMR spectra by applying a G-matrix defined as $\hat{G}(K) = \left[\begin{bmatrix} 1 & i \\ 1-i \end{bmatrix} \otimes \ldots \otimes \begin{bmatrix} 1 & i \\ 1-i \end{bmatrix} \otimes \begin{bmatrix} 1 & i \end{bmatrix} \right]$, where $i = \sqrt{-1}$, under conditions effective to edit the chemical shift multiplet components in the time domain. Alternatively, the transforming can be carried out by applying a F-matrix defined as $\hat{F}(K) = \hat{F}(K-1) \otimes \hat{F}(1)$, where $\hat{F}(1) = \begin{bmatrix} 1 & 1 \\ 1 & -1 \end{bmatrix}$, under conditions effective to edit the chemical shift multiplet components in the frequency domain.

[0061]   In an alternate embodiment, the method of conducting a $(N,N-K)$D GFT NMR experiment can further involve selecting m' indirect chemical shift evolution periods of the $(N-K)$D FT NMR experiment, where m' equals $K'+1$. Then, the m' indirect chemical shift evolution periods are jointly sampled. Next, NMR signals detected in a direct dimension are independently cosine and sine modulated to generate $(N-K-K')$D basic NMR spectra containing frequency domain signals with $2^{K'}$

chemical shift multiplet components, thereby enabling phase-sensitive sampling of all jointly sampled m' indirect chemical shift evolution periods. Finally, the $(N-K-K')$ D basic NMR spectra are transformed into $(N-K-K')$ D phase-sensitively edited basic NMR spectra, wherein the 2^K chemical shift multiplet components of the $(N-K-K')$ D basic NMR spectra are edited to yield $(N-K-K')$ D phase-sensitively edited basic NMR spectra having individual chemical shift multiplet components. The above-mentioned steps of selecting, jointly sampling, independently cosine and sine modulating, and transforming can be repeated one or more times, where m' is modified for each repetition.

[0062]   In an alternate embodiment, the method of conducting a $(N,N-K)$D GFT NMR experiment can further involve repeating one or more times the steps of selecting, jointly sampling, independently cosine and sine modulating, and transforming, where, for each repetition, the selecting involves selecting $m-j$ indirect chemical shift evolution periods out of the m indirect chemical shift evolution periods, wherein j ranges from 1 to K, under conditions effective to generate $2^{K-j}j$th order central peak NMR spectra.

[0063]   The method of conducting a $(N,N-K)$D GFT NMR experiment can also involve applying radiofrequency pulses of N-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety). Alternatively, the method can involve applying radiofrequency pulses of N-dimensional transverse relaxation optimized spectroscopy (TROSY) (Pervushin et al., Proc. Natl. Acad. Sci. USA, 94:12366-12371 (1997), which is hereby incorporated by reference in its entirety). In addition, the method can involve applying radiofrequency pulses so that spin-spin couplings are measured (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety). The spin-spin couplings can be residual dipolar spin-spin coupling constants (Bax, Protein Sci., 12:1-16 (2003), which is hereby incorporated by reference in its entirety). The method can also involve applying radiofrequency pulses so that nuclear spin relaxation times are measured by sampling nuclear spin relaxation delays (Palmer, Annu. Rev. Biophys. Biomol. Struct., 30:129-155 (2001),

which is hereby incorporated by reference in its entirety). The spin relaxation delays can be further jointly sampled with chemical shift evolution periods (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety). In another embodiment, the jointly sampling the m indirect chemical shift evolution periods is achieved with a single continuous acquisition (Frydman et al., Proc. Natl. Acad. Sci.,U.S.A., 99:15858-15862 (2002), which is hereby incorporated by reference in its entirety).

[0064]   The present invention also discloses specific GFT NMR experiments and different combinations of those experiments which allows one to obtain sequential backbone chemical shift assignments for determining the secondary structure of a protein molecule and complete assignments of chemical shift values for a protein molecule including aliphatic and aromatic sidechain spin systems.

Specific GFT NMR Experiments

[0065]   The present invention discloses the following six $(N,N-K)D$ GFT NMR experiments for the assignment of polypeptide backbone and $^{13}C^{\beta}$ resonance: (i) with $K = 3$, (5,2)D [HACACONHN] GFT NMR experiment and (5,2)D [HACA,CONHN] GFT NMR experiment for sequential assignment, (ii) with $K = 2$, (5,3)D [HACA,CONHN] GFT NMR experiment and (5,3)D [HACACONHN] GFT NMR experiment, where, in contrast to the (5,2)D experiments in (i), the ^{15}N chemical shifts evolve separately, and (iii) with $K = 1$, (4,3)D [CBCACONHN] GFT NMR experiment and (4,3)D [CBCA,CONHN] GFT NMR. The underlined letters indicate which chemical shifts that are jointly sampled. After G-matrix transformation, one obtains $2^{3+1} - 1 = 15$ 2D planes for the (5,2)D experiments ($K = 3$), seven 3D spectra for the (5,3)D experiments ($K = 2$) and three 3D spectra for the (4,3)D experiments ($K = 1$). Figure 4 illustrates the magnetization transfer pathways of the specific embodiments of these six GFT NMR experiments. (5,2)D [HACA,CONHN] / (5,2)D [HACACONHN] GFT NMR experiments and (5,3)D [HACA,CONHN] / (5,3)D [HACACONHN] GFT NMR experiments correlate the backbone amide ^{15}N and ^{1}HN chemical shifts of residue i with the $^{13}C'$, $^{13}C^{\alpha}$ and $^{1}H^{\alpha}$ chemical shifts of residue i-1 and i, respectively, via one-bond scalar couplings (Figures 4A-B). In addition, the often smaller two-bond scalar couplings between the $^{15}N_i$ and $^{13}C^{\alpha}_{i-1}$ may yield

sequential connectivities in the HACA,CONHN experiments. The comma separating "CA" from "CO" indicates that the intraresidue $^{13}C'$ chemical shift is obtained by creating two-spin coherence involving $^{13}C^{\alpha}$ and $^{13}C'$ during the intraresidue polarization transfer from $^{13}C^{\alpha}$ to ^{15}N (Löhr et al., J. Biomol. NMR 6:189–197 (1995), which is hereby incorporated by reference in its reference). (4,3)D [CBCACONHN] and (4,3)D [CBCA,CONHN] GFT NMR experiments correlate the backbone amide ^{15}N and ^{1}HN chemical shifts of residue i with the $^{13}C'$, $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ chemical shifts of residue i-1 and i, respectively, via one-bond scalar couplings (Figure 4C), and the often smaller two-bond scalar couplings between the $^{15}N_i$ and $^{13}C^{\alpha}_{i-1}$ may yield additional sequential connectivities in (4,3)D [CBCA,CONHN].

[0066]   Thus, the present invention relates to the above method of conducting a $(N,N-K)$D GFT NMR experiment, where N equals 5 and K equals 3 to conduct a (5,2)D [HACACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) an α-proton of amino acid residue i-1, $^{1}H^{\alpha}_{i-1}$; (2) an α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$; (3) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$; (4) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (5) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_i$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, $^{1}H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C'_{i-1}$, and $^{15}N_i$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H^{\alpha}_{i-1}, ^{13}C^{\alpha}_{i-1}, ^{13}C'_{i-1}, ^{15}N_i)$. One specific embodiment of this method ((5,2)D HACACONHN) involves applying radiofrequency pulses for a 5D FT NMR experiment according to the scheme shown in Figure 6.

[0067]   The present invention also relates to the above method of conducting a $(N,N-K)$D GFT NMR experiment, where N equals 5 and K equals 3 to conduct a (5,2)D [HACA,CONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having an amino acid residue, i, and the chemical shift values for the following nuclei are measured: (1) an α-proton of amino acid residue i-1, $^{1}H^{\alpha}_{i-1}$; (2) an α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$; (3) a polypeptide backbone carbonyl

carbon of amino acid residue i-1, $^{13}C'_{i-1}$; (4) a polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$; and (5) a polypeptide backbone amide proton of amino acid residue i-1, $^{1}H^{N}_{i-1}$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, $^{1}H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C'_{i-1}$, and $^{15}N_{i-1}$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H^{\alpha}_{i-1}, {}^{13}C^{\alpha}_{i-1}, {}^{13}C'_{i-1}, {}^{15}N_{i-1})$. One specific embodiment of this method ((5,2)D HACA,CONHN) involves applying radiofrequency pulses for a 5D FT NMR experiment according to the scheme shown in Figure 7A.

[0068]   Another aspect of the present invention relates to the above method of conducting a $(N,N\text{-}K)$D GFT NMR experiment, where N equals 5 and K equals 2 to conduct a (5,3)D [HACACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) an α-proton of amino acid residue i-1, $^{1}H^{\alpha}_{i-1}$; (2) an α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$; (3) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$; (4) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_{i}$; and (5) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_{i}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^{1}H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H^{\alpha}_{i-1}, {}^{13}C^{\alpha}_{i-1}, {}^{13}C'_{i-1})$.

[0069]   Yet another aspect of the present invention relates to the above method of conducting a $(N,N\text{-}K)$D GFT NMR experiment, where N equals 5 and K equals 2 to conduct a (5,3)D [HACA,CONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having an amino acid residue, i-1, and the chemical shift values for the following nuclei are measured: (1) an α-proton of amino acid residue i-1, $^{1}H^{\alpha}_{i-1}$; (2) an α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$; (3) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$; (4) a polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$; and (5) a polypeptide backbone amide proton of amino acid residue i-1, $^{1}H^{N}_{i-1}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^{1}H^{\alpha}_{i-1}$,

$^{13}C^{\alpha}{}_{i\text{-}1}$, and $^{13}C'{}_{i\text{-}1}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H^{\alpha}{}_{i\text{-}1}, {}^{13}C^{\alpha}{}_{i\text{-}1}, {}^{13}C'{}_{i\text{-}1})$.

[0070]   A further aspect of the present invention relates to the above method of conducting a $(N,N\text{-}K)D$ GFT NMR experiment, where N equals 4 and K equals 1 to conduct a (4,3)D [CBCACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, $i\text{-}1$ and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue $i\text{-}1$, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$; (2) a polypeptide backbone carbonyl carbon of amino acid residue $i\text{-}1$, $^{13}C'{}_{i\text{-}1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^1H^N{}_i$, (b) the selecting involves selecting 2 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$ and $^{13}C'{}_{i\text{-}1}$, and (c) the jointly sampling involves jointly sampling the 2 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C'{}_{i\text{-}1})$. One specific embodiment of this method ((4,3)D CBCACONHN) involves applying radiofrequency pulses for a 4D FT NMR experiment according to the scheme shown in Figure 8.

[0071]   The present invention also relates to the above method of conducting a $(N,N\text{-}K)D$ GFT NMR experiment, where N equals 4 and K equals 1 to conduct a (4,3)D [CBCA,CONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having an amino acid residue, $i\text{-}1$, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue $i\text{-}1$, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$; (2) a polypeptide backbone carbonyl carbon of amino acid residue $i\text{-}1$, $^{13}C'{}_{i\text{-}1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue $i\text{-}1$, $^{15}N_{i\text{-}1}$; and (4) a polypeptide backbone amide proton of amino acid residue $i\text{-}1$, $^1H^N{}_{i\text{-}1}$, (b) the selecting involves selecting 2 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$ and $^{13}C'{}_{i\text{-}1}$, and (c) the jointly sampling involves jointly sampling the 2 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C'{}_{i\text{-}1})$. One specific embodiment of this method ((4,3)D CBCA,CONHN) involves applying radiofrequency pulses for a 4D FT NMR experiment according to the scheme shown in Figure 7B.

[0072]   In addition, the present invention discloses the following GFT NMR experiments for the assignment of polypeptide backbone and sidechain resonances: (i) (4,3)D [HNN<u>CACBCA</u>] GFT NMR experiment, (ii) (4,3)D [<u>CBCACA</u>(CO)NHN]/(4,3)D [HNN(CO)<u>CACBCA</u>] GFT NMR experiments, (iii) (5,3)D [<u>HBHACBCACA</u>(CO)NHN] GFT NMR experiment, (iv) (5,3)D [<u>HCC</u>,CH-COSY] GFT NMR experiment, (v) (5,3)D [<u>HBCBCGCD</u>HD] GFT NMR experiment, (vi) (4,2)D [<u>HCC</u>H-COSY] GFT NMR experiment, and (vii) (5,2)D [<u>HCCC</u>H-COSY] GFT NMR experiment. Experiment (i) and (ii)/(iii) form pairs to sequentially assign backbone $^{13}C^\alpha$ and $^{13}C^\beta$ resonances. Experiment (iii) also provides $^1H^{\alpha/\beta}$ chemical shifts. The $^{13}C^{\alpha/\beta}$ and $^1H^{\alpha/\beta}$ chemical shifts, in turn, allow one to assign more peripheral spins of the aliphatic side-chain of a given amino acid residue using experiment (iv). Experiments (v) and (vi) can be used for resonance assignments of aromatic side-chain spins. The assignment of the side-chain chemical shifts can be further supported with experiment (vii). The magnetization transfer pathways of specific embodiments of these GFT NMR experiments (i)-(vii) are depicted in Figures 5A-G, respectively.

[0073]   Thus, the present invention also relates to the above method of conducting a (N,N-K)D GFT NMR experiment, where N equals 4 and K equals 1 to conduct a (4,3)D [HNN<u>CACBCA</u>] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, $i-1$ and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residues i and $i-1$, $^{13}C^{\alpha/\beta}_{i/i-1}$; (2) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (3) a polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$, (b) the selecting involves selecting 2 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}_{i/i-1}$ and $^{13}C^\alpha_{i/i-1}$, and (c) the jointly sampling involves jointly sampling the 2 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}_{i/i-1}, {}^{13}C^\alpha_{i/i-1})$. One specific embodiment of this method ((4,3)D HNN<u>CACBCA</u>) involves applying radiofrequency pulses for a 4D FT NMR experiment according to the scheme shown in Figure 9.

[0074]   In an alternate embodiment, the above method can be modified, where N equals 4 and K equals 2, to conduct a (4,2)D [HN<u>NCACBCA</u>] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two

consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residues i and i-1, $^{13}C^{\alpha/\beta}{}_{i/i-1}$; (2) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (3) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i/i-1}$, $^{13}C^{\alpha}{}_{i/i-1}$, and $^{15}N_i$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i/i-1}, {}^{13}C^{\alpha}{}_{i/i-1}, {}^{15}N_i)$.

[0075]   In another alternate embodiment, the above method can be modified, where N equals 4 and K equals 1 to conduct a (4,3)D [HNN(CO)CACBCA] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$; (2) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (3) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_i$, (b) the selecting involves selecting 2 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i-1}$ and $^{13}C^{\alpha}{}_{i-1}$, and (c) the jointly sampling involves jointly sampling the 2 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i-1}, {}^{13}C^{\alpha}{}_{i-1})$. One specific embodiment of this method ((4,3)D HNN(CO)CACBCA) involves applying radiofrequency pulses for a 4D FT NMR experiment according to the scheme shown in Figure 10.

[0076]   In yet another alternate embodiment, the above method can be modified, where N equals 4 and K equals 2 to conduct a (4,2)D [HNN(CO)CACBCA] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$; (2) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (3) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i-1}$, $^{13}C^{\alpha}{}_{i-1}$, and $^{15}N_i$; and (c) the jointly sampling involves jointly sampling the 3

chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}_{i-1}, ^{13}C^{\alpha}_{i-1}, ^{15}N_i)$.

[0077] In another alternate embodiment, the above method can be modified, where N equals 5 and K equals 2 to conduct a (5,3)D [HNN<u>COCACBCA</u>] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$; (2) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^{13}C^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$, (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}_{i-1}, ^{13}C^{\alpha}_{i-1}, ^{13}C'_{i-1})$.

[0078] In yet another alternate embodiment, the above method can be modified, where N equals 5 and K equals 3 to conduct a (5,2)D [HN<u>NCOCACBCA</u>] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$; (2) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_i$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, $^{13}C^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C'_{i-1}$, and $^{15}N_i$; and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}_{i-1}, ^{13}C^{\alpha}_{i-1}, ^{13}C'_{i-1}, ^{15}N_i)$.

[0079] Another aspect of the present invention relates to the above method of conducting a $(N,N-K)$D GFT NMR experiment, where N equals 4 and K equals 1 to conduct a (4,3)D [<u>CBCACA</u>(CO)NHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-

carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$; (2) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (3) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_i$, (b) the selecting involves selecting 2 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i-1}$ and $^{13}C^{\alpha}{}_{i-1}$, and (c) the jointly sampling involves jointly sampling the 2 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i-1}, ^{13}C^{\alpha}{}_{i-1})$. One specific embodiment of this method ((4,3)D CBCACA(CO)NHN) involves applying radiofrequency pulses for a 4D FT NMR experiment according to the scheme shown in Figure 11.

[0080] In an alternate embodiment, the above method can be modified, where N equals 4 and K equals 2 to conduct a (4,2)D [CBCACA(CO)NHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$; (2) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (3) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 4D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i-1}$, $^{13}C^{\alpha}{}_{i-1}$, and $^{15}N_i$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i-1}, ^{13}C^{\alpha}{}_{i-1}, ^{15}N_i)$.

[0081] In another alternate embodiment, the above method can be modified, where N equals 5 and K equals 2 to conduct a (5,3)D [CBCACACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i-1}$; (2) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'{}_{i-1}$, (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^{13}C^{\alpha/\beta}{}_{i-1}$, $^{13}C^{\alpha}{}_{i-1}$, and $^{13}C'{}_{i-1}$, (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}{}_{i-1}, ^{13}C^{\alpha}{}_{i-1}, ^{13}C'{}_{i-1})$.

[0082]   In yet another alternate embodiment, the above method can be modified, where N equals 5 and K equals 3 to conduct a (5,2)D [CBCACACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two consecutive amino acid residues, i-1 and i, and the chemical shift values for the following nuclei are measured: (1) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$; (2) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, $^{13}C^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C'_{i-1}$, and $^{15}N_i$; (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{13}C^{\alpha/\beta}_{i-1}, ^{13}C^{\alpha}_{i-1}, ^{13}C'_{i-1}, ^{15}N_i)$.

[0083]   Another aspect of the present invention relates to the above method of conducting a $(N,N-K)$D GFT NMR experiment, where N equals 5 and K equals 2 to conduct a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two amino acid residues, i and i-1, and the chemical shift values for the following nuclei are measured: (1) α- and β- protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$; (2) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^1H^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha/\beta}_{i-1}$, and $^{13}C^{\alpha}_{i-1}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H^{\alpha/\beta}_{i-1}, ^{13}C^{\alpha/\beta}_{i-1}, ^{13}C^{\alpha}_{i-1})$. One specific embodiment of this method ((5,3)D HBHACBCACA(CO)NHN) involves applying radiofrequency pulses for a 5D FT NMR experiment according to the scheme shown in Figure 12.

[0084]   In an alternate embodiment, the above method can be modified, where N equals 6 and K equals 3 to conduct a (6,3)D [HBHACBCACACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two amino acid residues, i and i-1, and the chemical shift values for the following nuclei are measured: (1) α- and β protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$; (2) α- and β-

carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$; (3) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'{}_{i\text{-}1}$; (4) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (5) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_{i}$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 6D FT NMR experiment, $^{1}H^{\alpha/\beta}{}_{i\text{-}1}$, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$, $^{13}C^{\alpha}{}_{i\text{-}1}$, and $^{13}C'{}_{i\text{-}1}$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C^{\alpha}{}_{i\text{-}1}, {}^{13}C'{}_{i\text{-}1})$.

[0085]   In another alternate embodiment, the above method can be modified, where N equals 5 and K equals 3 to conduct a (5,2)D [HBHACBCACA(CO)NHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two amino acid residues, i and i-1, and the chemical shift values for the following nuclei are measured: (1) α- and β protons of amino acid residue i-1, $^{1}H^{\alpha/\beta}{}_{i\text{-}1}$; (2) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$; (3) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (4) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_{i}$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, $^{1}H^{\alpha/\beta}{}_{i\text{-}1}$, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$, $^{13}C^{\alpha}{}_{i\text{-}1}$, and $^{15}N_i$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C^{\alpha}{}_{i\text{-}1}, {}^{15}N_i)$.

[0086]   In yet another alternate embodiment, the above method can be modified, where N equals 6 and K equals 4 to conduct a (6,2)D [HBHACBCACACONHN] GFT NMR experiment. In this method, (a) the sample is a protein molecule having two amino acid residues, i and i-1, and the chemical shift values for the following nuclei are measured: (1) α- and β protons of amino acid residue i-1, $^{1}H^{\alpha/\beta}{}_{i\text{-}1}$; (2) α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$; (3) a polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'{}_{i\text{-}1}$; (4) a polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$; and (5) a polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}{}_{i}$, (b) the selecting involves selecting 5 chemical shift evolution periods of the 6D FT NMR experiment, $^{1}H^{\alpha/\beta}{}_{i\text{-}1}$, $^{13}C^{\alpha/\beta}{}_{i\text{-}1}$, $^{13}C^{\alpha}{}_{i\text{-}1}$, $^{13}C'{}_{i\text{-}1}$, and $^{15}N_i$, and (c) the jointly sampling involves jointly sampling the 5 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C^{\alpha/\beta}{}_{i\text{-}1}, {}^{13}C^{\alpha}{}_{i\text{-}1}, {}^{13}C'{}_{i\text{-}1}, {}^{15}N_i)$.

[0087] Yet another aspect of the present invention relates to the above method of conducting a $(N,N-K)$D GFT NMR experiment, where N equals 5 and K equals 2 to conduct a (5,3)D [HCC,CH-COSY] GFT NMR experiment. In this method, (a) the chemical shift values for the following nuclei are measured: (1) a proton, ^1H; (2) a carbon coupled to ^1H, ^{13}C; and (3) a carbon coupled to ^{13}C, $^{13}C^{coupled}$; and (4) a proton coupled to $^{13}C^{coupled}$, $^1H^{coupled}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, ^1H, ^{13}C, and $^{13}C^{coupled}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H,^{13}C,^{13}C^{coupled})$. The sample in this method can be any molecule such as (metallo)-organic molecules and complexes, nucleic acid molecules such as DNA and RNA, lipids, or polymers. In one embodiment, the chemical shift evolution periods for ^{13}C and $^{13}C^{coupled}$ can be correlated using total correlation spectroscopy (TOCSY) (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety). In another embodiment, (a) the sample is a protein molecule having an amino acid residue, i, and the chemical shift values for the following nuclei are measured: (1) a proton of amino acid residue i, ^1H_i; (2) a carbon of amino acid residue i coupled to ^1H_i, $^{13}C_i$; and (3) a carbon coupled to $^{13}C_i$, $^{13}C_i^{coupled}$; and (4) a proton coupled with $^{13}C_i^{coupled}$, $^1H_i^{coupled}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, ^1H_i, $^{13}C_i$, and $^{13}C_i^{coupled}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H_i,^{13}C_i,^{13}C_i^{coupled})$. One specific embodiment of this method ((5,3)D HCC,CH-COSY) involves applying radiofrequency pulses for a 5D FT NMR experiment according to the scheme shown in Figure 13.

[0088] The present invention also relates to the above method of conducting a $(N,N-K)$D GFT NMR experiment, where N equals 5 and K equals 2 to conduct a (5,3)D [HBCBCGCDHD] GFT NMR experiment. In this method, (a) the sample is a protein molecule having an amino acid residue, i, with an aromatic side chain, and the chemical shift values for the following nuclei are measured: (1) a β–proton of amino acid residue i, $^1H^\beta_i$; (2) a β-carbon of amino acid residue i, $^{13}C^\beta_i$; (3) a γ-carbon of amino acid residue i, $^{13}C^\gamma_i$; (4) a δ-carbon of amino acid residue i, $^{13}C^\delta_i$; and (5) a

δ–proton of amino acid residue i, $^1H^\delta_i$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^1H^\beta_i$, $^{13}C^\beta_i$, and $^{13}C^\delta_i$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H^\beta_i, {}^{13}C^\beta_i, {}^{13}C^\delta_i)$. One specific embodiment of this method ((5,3)D <u>HBCB</u>CG<u>C</u>DHD) involves applying radiofrequency pulses for a 5D FT NMR experiment according to the scheme shown in Figure 14.

[0089]   In an alternate embodiment, the above method can be modified, where N equals 5 and K equals 3 to conduct a (5,2)D [<u>H</u>BCBCGCDHD] GFT NMR experiment. In this method, (a) the sample is a protein molecule having an amino acid residue, i, with an aromatic side chain, and the chemical shift values for the following nuclei are measured: (1) a β–proton of amino acid residue i, $^1H^\beta_i$; (2) a β-carbon of amino acid residue i, $^{13}C^\beta_i$; (3) a γ-carbon of amino acid residue i; (4) a δ-carbon of amino acid residue i, $^{13}C^\delta_i$; and (5) a δ–proton of amino acid residue i, $^1H^\delta_i$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, $^1H^\beta_i$, $^{13}C^\beta_i$, $^{13}C^\gamma_i$, and $^{13}C^\delta_i$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H^\beta_i, {}^{13}C^\beta_i, {}^{13}C^\gamma_i, {}^{13}C^\delta_i)$.

[0090]   Another aspect of the present invention relates to the above method of conducting a $(N,N\text{-}K)D$ GFT NMR experiment, where N equals 4 and K equals 2 to conduct a (4,2)D [<u>H</u>C<u>C</u>H-COSY] GFT NMR experiment. In this method, (a) the chemical shift values for the following nuclei are measured: (1) a proton, ^1H; (2) a carbon coupled to ^1H, ^{13}C; (3) a carbon coupled to ^{13}C, $^{13}C^{coupled}$; and (4) a proton coupled to $^{13}C^{coupled}$, $^1H^{coupled}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 4D FT NMR experiment, ^1H, ^{13}C, and $^{13}C^{coupled}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H, {}^{13}C, {}^{13}C^{coupled})$. The sample in this method can be any molecule such as (metallo)-organic molecules and complexes, nucleic acid molecules such as DNA and RNA, lipids, or polymers. In one embodiment, the chemical shift evolution periods for ^{13}C and $^{13}C^{coupled}$ are correlated using total correlation spectroscopy (TOCSY). In another embodiment, (a) the sample is a protein molecule having an amino acid residue, i, and the chemical shift values for the following nuclei are measured: (1) a proton of amino acid residue i, ^1H_i; (2) a carbon

of amino acid residue i coupled to ^1H_i, $^{13}C_i$; (3) a carbon coupled to $^{13}C_i$, $^{13}C_i^{coupled}$; and (4) a proton coupled to $^{13}C_i^{coupled}$, $^1H_i^{coupled}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 4D FT NMR experiment, ^1H_i, $^{13}C_i$, and $^{13}C_i^{coupled}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H_i, ^{13}C_i, ^{13}C_i^{coupled})$. One specific embodiment of this method ((4,2)D H<u>CC</u>H-COSY) involves applying radiofrequency pulses for a 4D FT NMR experiment according to the scheme shown in Figure 15.

[0091]   Yet another aspect of the present invention relates to the above method of conducting a (N,N-K)D GFT NMR experiment, where N equals 5 and K equals 3 to conduct a (5,2)D [H<u>CCC</u>H-COSY] GFT NMR experiment. In this method, (a) the chemical shift values for the following nuclei are measured: (1) a proton ^1H; (2) a carbon coupled to ^1H, ^{13}C; (3) a carbon coupled to ^{13}C, $^{13}C^{coupled}$; (4) a carbon coupled to $^{13}C^{coupled}$, $^{13}C^{coupled-2}$; and (5) a proton coupled with $^{13}C^{coupled-2}$, $^1H^{coupled-2}$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, ^1H, ^{13}C, $^{13}C^{coupled}$, and $^{13}C^{coupled-2}$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H, ^{13}C, ^{13}C^{coupled}, ^{13}C^{coupled-2})$. The sample in this method can be any molecule such as (metallo)-organic molecules and complexes, nucleic acid molecules such as DNA and RNA, lipids, or polymers. In one embodiment, (a) the sample is a protein molecule having an amino acid residue, i, and the chemical shift values for the following nuclei are measured: (1) a proton of amino acid residue i, ^1H_i; (2) a carbon of amino acid residue i coupled to ^1H_i, $^{13}C_i$; (3) a carbon coupled to $^{13}C_i$, $^{13}C_i^{coupled}$; (4) a carbon coupled to $^{13}C_i^{coupled}$, $^{13}C_i^{coupled-2}$; and (5) a proton coupled with $^{13}C_i^{coupled-2}$, $^1H_i^{coupled-2}$, (b) the selecting involves selecting 4 chemical shift evolution periods of the 5D FT NMR experiment, ^1H_i, $^{13}C_i$, $^{13}C_i^{coupled}$, and $^{13}C_i^{coupled-2}$, and (c) the jointly sampling involves jointly sampling the 4 chemical shift evolution periods in an indirect time domain dimension, $t_1(^1H_i, ^{13}C_i, ^{13}C_i^{coupled}, ^{13}C_i^{coupled-2})$.

[0092]   In an alternate embodiment, the above method can be modified, where N equals 5 and K equals 3 to conduct a (5,3)D [H<u>CCC</u>H-COSY] GFT NMR experiment. In this method, (a) the chemical shift values for the following nuclei are measured: (1) a proton, ^1H; (2) a carbon coupled to ^1H, ^{13}C; (3) a carbon coupled to

^{13}C, $^{13}C^{coupled}$; (4) a carbon coupled to $^{13}C^{coupled}$, $^{13}C^{coupled-2}$; and (5) a proton coupled with $^{13}C^{coupled-2}$, $^{1}H^{coupled-2}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, ^{1}H, ^{13}C, and $^{13}C^{coupled}$, and (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H,^{13}C,^{13}C^{coupled})$. The sample in this method can be any molecule such as (metallo)-organic molecules and complexes, nucleic acid molecules such as DNA and RNA, lipids, or polymers. In another embodiment, (a) the sample is a protein molecule having an amino acid residue, i, and the chemical shift values for the following nuclei are measured: (1) a proton of amino acid residue i, $^{1}H_i$; (2) a carbon of amino acid residue i coupled to $^{1}H_i$, $^{13}C_i$; (3) a carbon coupled to $^{13}C_i$, $^{13}C_i^{coupled}$; (4) a carbon coupled to $^{13}C_i^{coupled}$, $^{13}C_i^{coupled-2}$; and (5) a proton coupled with $^{13}C_i^{coupled-2}$, $^{1}H_i^{coupled-2}$, (b) the selecting involves selecting 3 chemical shift evolution periods of the 5D FT NMR experiment, $^{1}H_i$, $^{13}C_i$, and $^{13}C_i^{coupled}$, (c) the jointly sampling involves jointly sampling the 3 chemical shift evolution periods in an indirect time domain dimension, $t_1(^{1}H_i,^{13}C_i,^{13}C_i^{coupled})$.

Combinations of GFT NMR Experiments

[0093]   A set of multidimensional GFT NMR experiments enables one to devise strategies for GFT NMR-based (high throughput) resonance assignment of proteins or other molecules.

[0094]   Thus, another aspect of the present invention relates to a method for sequentially assigning chemical shift values of an α-proton, $^{1}H^{\alpha}$, an α-carbon, $^{13}C^{\alpha}$, a polypeptide backbone carbonyl carbon, ^{13}C', a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^{1}H^{N}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,2)D [HACACONHN] GFT NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i-1, $^{1}H^{\alpha}_{i-1}$, the α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$, the polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_i$ and (2) a (5,2)D [HACA,CONHN] GFT NMR experiment to measure

and connect the chemical shift values of $^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^1H^N_{i-1}$. Then, sequential assignments of the chemical shift values of $^1H^\alpha$, $^{13}C^\alpha$, $^{13}C'$, ^{15}N, and $^1H^N$ are obtained

5   by (i) matching the chemical shift values of $^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,2)D [<u>HACACONHN</u>] GFT NMR experiment with the chemical shift values of $^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,2)D [<u>HACA,CONHN</u>] GFT NMR experiment, (ii) using the chemical shift values of $^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, and $^{13}C'_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, <u>NMR of Proteins and Nucleic Acids,</u>

10   Wiley, New York (1986); Grzesiek et al., <u>J. Biomol. NMR</u>, 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements (such as α-helices and β-sheets) within the polypeptide chain (Spera et al., <u>J. Am. Chem. Soc.</u>, 113:5490-5492 (1991); Wishart et al., <u>Biochemistry</u>, 31:1647-1651

15   (1992), which are hereby incorporated by reference in their entirety).

[0095]   Yet another aspect of the present invention relates to a method for sequentially assigning chemical shift values of an α-proton, $^1H^\alpha$, an α-carbon, $^{13}C^\alpha$, a polypeptide backbone carbonyl carbon, $^{13}C'$, a polypeptide backbone amide nitrogen,

20   ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,3)D [<u>HACACO</u>NHN] GFT NMR experiment to measure and connect the chemical shift values of the α-proton of amino acid residue i-1, $^1H^\alpha_{i-1}$,

25   the α-carbon of amino acid residue i-1, $^{13}C^\alpha_{i-1}$, the polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$ and (2) a (5,3)D [<u>HACA,CO</u>NHN] GFT NMR experiment to measure and connect the chemical shift values of $^1H^\alpha_{i-1}$, $^{13}C^\alpha_{i-1}$, $^{13}C'_{i-1}$, the polypeptide

30   backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^1H^N_{i-1}$. Then, sequential assignments of the chemical shift values of $^1H^\alpha$, $^{13}C^\alpha$, $^{13}C'$, ^{15}N, and $^1H^N$ are obtained

by (i) matching the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,3)D [HACACONHN] GFT NMR experiment with the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ measured by the (5,3)D [HACA,CONHN] GFT NMR experiment, (ii) using the chemical shift values of $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, and $^{13}C'_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986); Grzesiek et al., J. Biomol. NMR, 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements (such as α-helices and β-sheets) within the polypeptide chain (Spera et al., J. Am. Chem. Soc., 113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0096]   A further aspect of the present invention relates to a method for sequentially assigning chemical shift values of α- and β-carbons, $^{13}C^{\alpha/\beta}$, a polypeptide backbone carbonyl carbon, $^{13}C'$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^1H^N$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (4,3)D [CBCACONHN] GFT NMR experiment to measure and connect the chemical shift values of the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$, the polypeptide backbone carbonyl carbon of amino acid residue i-1, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_i$, and the polypeptide backbone amide proton of amino acid residue i, $^1H^N_i$ and (2) a (4,3)D [CBCA,CONHN] GFT NMR experiment to measure and connect the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$, $^{13}C'_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^1H^N_{i-1}$. Then, sequential assignments of the chemical shift values of $^{13}C^{\alpha/\beta}$, $^{13}C'$, ^{15}N, and $^1H^N$ are obtained by (i) matching the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$ and $^{13}C'_{i-1}$ measured by the (4,3)D [CBCACONHN] GFT NMR experiment with the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$ and $^{13}C'_{i-1}$ measured by the (4,3)D [CBCA,CONHN] GFT NMR experiment, (ii) using the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$ and $^{13}C'_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, NMR of Proteins and Nucleic

Acids, Wiley, New York (1986); Grzesiek et al., J. Biomol. NMR, 3: 185-204 (1993), which are hereby incorporated by reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements (such as α-helices and β-sheets) within the polypeptide chain (Spera et al., J. Am. Chem. Soc., 113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0097]   The present invention also relates to a method for sequentially assigning chemical shift values of α- and β-carbons, $^{13}C^{\alpha/\beta}$, a polypeptide backbone amide nitrogen, ^{15}N, and a polypeptide backbone amide proton, $^{1}H^{N}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (4,3)D [HNNCACBCA] GFT NMR experiment to measure and connect the chemical shift values of the α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$, the α-carbon of amino acid residue i-1, $^{13}C^{\alpha}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i-1, $^{15}N_{i-1}$, and the polypeptide backbone amide proton of amino acid residue i-1, $^{1}H^{N}_{i-1}$ and (2) a GFT NMR experiment selected from the group consisting of a (4,3)D [HNN(CO)CACBCA] GFT NMR experiment, a (4,3)D [CBCACA(CO)NHN] GFT NMR experiment , and a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, the polypeptide backbone amide nitrogen of amino acid residue i, $^{15}N_{i}$, and the polypeptide backbone amide proton of amino acid residue i, $^{1}H^{N}_{i}$. Then, sequential assignments of the chemical shift values of $^{13}C^{\alpha/\beta}$, ^{15}N, and $^{1}H^{N}$ are obtained by (i) matching the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$ measured by the GFT NMR experiment selected from the group consisting of a (4,3)D [HNN(CO)CACBCA] GFT NMR experiment, a (4,3)D [CBCACA(CO)NHN] GFT NMR experiment , and a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment with the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$ measured by the (4,3)D [HNNCACBCA] GFT NMR experiment, (ii) using the chemical shift values of $^{13}C^{\alpha/\beta}_{i-1}$ to identify the type of amino acid residue i-1 (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986); Grzesiek et al., J. Biomol. NMR, 3: 185-204 (1993), which are hereby incorporated by

reference in their entirety), and (iii) mapping sets of sequentially connected chemical shift values to the amino acid sequence of the polypeptide chain and using the chemical shift values to locate secondary structure elements (such as α-helices and β-sheets) within the polypeptide chain (Spera et al., J. Am. Chem. Soc., 113:5490-5492 (1991); Wishart et al., Biochemistry, 31:1647-1651 (1992), which are hereby incorporated by reference in their entirety).

[0098]   Another aspect of the present invention relates to a method for assigning chemical shift values of γ-, δ-, and ε-aliphatic sidechain protons, $^1H^{\gamma/\delta/\varepsilon}$, and chemical shift values of γ-, δ-, and ε-aliphatic sidechain carbons located peripheral to β-carbons, $^{13}C^{\gamma/\delta/\varepsilon}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,3)D [HCC,CH-COSY] GFT NMR experiment to measure and connect the chemical shift values of a proton of amino acid residue i-1, $^1H_{i-1}$, a carbon of amino acid residue i-1 coupled to $^1H_{i-1}$, $^{13}C_{i-1}$, a carbon coupled to $^{13}C_{i-1}$, $^{13}C_{i-1}{}^{coupled}$, and a proton coupled to $^{13}C_{i-1}{}^{coupled}$, $^1H_{i-1}{}^{coupled}$, and (2) a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of α- and β-protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$, and α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$. Then, assignments of the chemical shift values of $^1H^{\gamma/\delta/\varepsilon}$ and $^{13}C^{\gamma/\delta/\varepsilon}$ are obtained by (i) identifying $^1H_{i-1}$, $^{13}C_{i-1}$, $^{13}C_{i-1}{}^{coupled}$, and $^1H_{i-1}{}^{coupled}$ measured by the (5,3)D [HCC,CH-COSY] GFT NMR experiment as $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C^{\beta}_{i-1}$, and $^1H^{\beta}_{i-1}$, respectively, and thereby matching the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ with the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ measured by the (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment, and (ii) using the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ in conjunction with other chemical shift connections from the (5,3)D [HCC,CH-COSY] GFT NMR experiment to measure the chemical shift values of $^1H^{\gamma/\delta/\varepsilon}_{i-1}$ and $^{13}C^{\gamma/\delta/\varepsilon}_{i-1}$.

[0099]   Yet another aspect of the present invention relates to a method for assigning chemical shift values of γ-, δ-, and ε-aliphatic sidechain protons, $^1H^{\gamma/\delta/\varepsilon}$, and chemical shift values of γ-, δ-, and ε-aliphatic sidechain carbons located peripheral to β-carbons, $^{13}C^{\gamma/\delta/\varepsilon}$, of a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear

magnetic resonance (NMR) experiments on the protein sample including: (1) a (4,2)D [HCCH-COSY] GFT NMR experiment to measure and connect the chemical shift values of a proton of amino acid residue i-1, $^1H_{i-1}$, a carbon of amino acid residue i-1 coupled to $^1H_{i-1}$, $^{13}C_{i-1}$, a carbon coupled to $^{13}C_{i-1}$, $^{13}C_{i-1}^{coupled}$, and a proton coupled to $^{13}C_{i-1}^{coupled}$, $^1H_{i-1}^{coupled}$, and (2) a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of α- and β-protons of amino acid residue i-1, $^1H^{\alpha/\beta}_{i-1}$, and α- and β-carbons of amino acid residue i-1, $^{13}C^{\alpha/\beta}_{i-1}$. Then, assignments of the chemical shift values of $^1H^{\gamma/\delta/\varepsilon}$ and $^{13}C^{\gamma/\delta/\varepsilon}$ are obtained by (i) identifying $^1H_{i-1}$, $^{13}C_{i-1}$, $^{13}C_{i-1}^{coupled}$, and $^1H_{i-1}^{coupled}$ measured by the (4,2)D [HCCH-COSY] GFT NMR experiment as $^1H^{\alpha}_{i-1}$, $^{13}C^{\alpha}_{i-1}$, $^{13}C^{\beta}_{i-1}$, and $^1H^{\beta}_{i-1}$, respectively, and thereby matching the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ with the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ measured by the (5,3)D HBHACBCACA(CO)NHN] GFT NMR experiment, and (ii) using the chemical shift values of $^1H^{\alpha/\beta}_{i-1}$ and $^{13}C^{\alpha/\beta}_{i-1}$ in conjunction with other chemical shift connections from the (4,2)D [HCCH-COSY] GFT NMR experiment to measure the chemical shift values of $^1H^{\gamma/\delta/\varepsilon}_{i-1}$ and $^{13}C^{\gamma/\delta/\varepsilon}_{i-1}$.

[0100]   A further aspect of the present invention relates to a method for assigning chemical shift values of a γ-carbon, $^{13}C^{\gamma}$, a δ-carbon, $^{13}C^{\delta}$, and a δ-proton, $^1H^{\delta}$, of an amino acid residue containing an aromatic spin system in a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a (5,3)D [HBCBCGCDHD] GFT NMR experiment to measure and connect the chemical shift values of a β-proton of amino acid residue i-1, $^1H^{\beta}_{i-1}$, a β-carbon of amino acid residue i-1, $^{13}C^{\beta}_{i-1}$, a γ-carbon of amino acid residue i-1, $^{13}C^{\gamma}_{i-1}$, a δ-carbon of amino acid residue i-1, $^{13}C^{\delta}_{i-1}$, and a δ-proton of amino acid residue i-1, $^1H^{\delta}_{i-1}$, and (2) a (5,3)D [HBHACBCACA(CO)NHN] GFT NMR experiment to measure and connect the chemical shift values of $^1H^{\beta}_{i-1}$ and $^{13}C^{\beta}_{i-1}$. Then, assignments of the chemical shift values of $^{13}C^{\gamma}$, $^{13}C^{\delta}$, and $^1H^{\delta}$ are obtained by (i) matching the chemical shift values of $^1H^{\beta}_{i-1}$ and $^{13}C^{\beta}_{i-1}$ measured by the (5,3)D HBCBCACA(CO)NHN GFT NMR experiment with the chemical shift values of $^1H^{\beta}_{i-1}$ and $^{13}C^{\beta}_{i-1}$ measured by the (5,3)D [HBCBCGCDHD] GFT NMR

experiment, and (ii) using the chemical shift values of $^{13}C^\gamma$, $^{13}C^\delta$, and $^1H^\delta$ to identify the type of amino acid residue containing the aromatic spin system.

[0101]   The present invention also relates to a method for assigning chemical shift values of aliphatic and aromatic protons and aliphatic and aromatic carbons of an amino acid residue containing aliphatic and aromatic spin systems in a protein molecule. The method involves providing a protein sample and conducting a set of G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample including: (1) a first GFT NMR experiment, which is selected from the group consisting of a (5,3)D [HCC,CH-COSY] GFT NMR experiment, a (4,2)D [HCCH-COSY] GFT NMR experiment, a (5,2)D [HCCCH-COSY] GFT NMR experiment, and a (5,3)D [HCCCH-COSY] GFT NMR experiment and is acquired for the aliphatic spin system, to measure and connect the chemical shift values of α- and β-protons of amino acid residue i, $^1H^{\alpha/\beta}_i$, α- and β-carbons of amino acid residue i, $^{13}C^{\alpha/\beta}_i$, a γ-carbon of amino acid residue i, $^{13}C^\gamma_i$, and (2) a second GFT NMR experiment, which is selected from the group consisting of a (5,3)D [HCC,CH-COSY] GFT NMR experiment, a (4,2)D [HCCH-COSY] GFT NMR experiment, a (5,2)D [HCCCH-COSY] GFT NMR experiment, and a (5,3)D [HCCCH-COSY] GFT NMR experiment and is acquired for the aromatic spin system, to measure and connect the chemical shift values of $^{13}C^\gamma_i$ and other aromatic protons and carbons of amino acid residue i. Then, assignments of the chemical shift values of the aliphatic and aromatic protons and aliphatic and aromatic carbons are obtained by matching the chemical shift value of $^{13}C^\gamma_i$ measured by the first GFT NMR experiment with the chemical shift value of $^{13}C^\gamma_i$ measured by the second GFT NMR experiment. In another embodiment, the set of GFT NMR experiments can be conducted by using $^{13}C^\gamma$ steady state magnetization to generate first order central peaks.

[0102]   The above-described methods for assigning chemical shift values in a protein molecule can involve further subjecting the protein sample to nuclear Overhauser enhancement spectroscopy (NOESY) (Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York (1986), which is hereby incorporated by reference in its entirety), to NMR experiments that measure scalar coupling constants (Eberstadt et al., Angew. Chem. Int. Ed. Engl., 34:1671-1695 (1995); Cordier et al., J. Am.

<u>Chem. Soc.</u>, 121:1601-1602 (1999), which are hereby incorporated by reference in their entirety), or to NMR experiments that measure residual dipolar coupling constants (Prestegard, <u>Nature Struct. Biol.</u>, 5:517-522 (1998); Tjandra et al., <u>Science</u>, 278:1111-1114 (1997), which are hereby incorporated by reference in their entirety), to deduce the tertiary fold or tertiary structure of the protein molecule.

[0103]   Another aspect of the present invention relates to a method for obtaining assignments of chemical shift values of ^1H, ^{13}C, and ^{15}N of a protein molecule. The method involves providing a protein sample and conducting five G matrix Fourier transformation (GFT) nuclear magnetic resonance (NMR) experiments on the protein sample, where (1) a first experiment is a (4,3)D [HNN<u>CACBCA</u>] GFT NMR experiment for obtaining intraresidue correlations of chemical shift values; (2) a second experiment is a (5,3)D [<u>HBHACBCACA</u>(CO)NHN] GFT NMR experiment for obtaining interresidue correlations of chemical shift values; (3) a third experiment is a (5,3)D [<u>HCC</u>,CH-COSY] GFT NMR experiment for obtaining assignments of aliphatic sidechain chemical shift values; (4) a fourth experiment is a (5,3)D [<u>HBCBCGCDHD</u>] GFT NMR experiment for linking chemical shift values of aliphatic protons, $^1H^\beta$ and $^{13}C^\beta$, and aromatic protons, $^{13}C^\delta$ and $^1H^\delta$; and (5) a fifth experiment is a (4,2)D [<u>HCC</u>H-COSY] GFT NMR experiment for obtaining assignments of aromatic sidechain chemical shift values. These five GFT NMR experiments can be employed for obtaining nearly complete resonance assignments of proteins including aliphatic and aromatic side chain spin systems.

EXAMPLES

[0104]   The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Example 1 – Frequency Domain Editing of Chemical Shift Multiplets and Relation to the Formalism for Time Domain Editing

[0105]   When designing a GFT NMR experiment (Figure 1), one first identifies a "target" dimensionality, N_t, at which the majority of the peaks are resolved. The dimensionality N of a given FT NMR spectrum is then adapted to N_t by

jointly sampling $K + 1$ chemical shifts ($K = N - N_t$) in a hypercomplex manner, while $N_t - 1$ dimensions are sampled in a conventional fashion. As outlined, this yields 2^K N_tD spectra. As an example, the case of $K = 3$ in the frequency domain (Figures 2, 16, and 17) is described in this example. The indirect evolution time shall be t, and

5   Ω_0 shall be the chemical shift detected in quadrature in each of the N_tD spectra. Ω_1, Ω_2 and Ω_3 are the three jointly sampled shifts. The phases of the $90°$ pulses generating transverse magnetization for frequency labeling are chosen so that the transfer amplitudes of the real parts of the $2^K = 8$ spectra, Sjr ($j = 1... \ 8$) are proportional to:

10     $S1r \propto \quad \cos(\Omega_0 \, t) \cos(\Omega_1 \, t) \cos(\Omega_2 \, t) \cos(\Omega_3 \, t)$

$S2r \propto \quad \cos(\Omega_0 \, t) \sin(\Omega_1 \, t) \cos(\Omega_2 \, t) \cos(\Omega_3 \, t)$

$S3r \propto \quad \cos(\Omega_0 \, t) \cos(\Omega_1 \, t) \sin(\Omega_2 \, t) \cos(\Omega_3 \, t)$

$S4r \propto \quad \cos(\Omega_0 \, t) \sin(\Omega_1 \, t) \sin(\Omega_2 \, t) \cos(\Omega_3 \, t)$

$S5r \propto \quad \cos(\Omega_0 \, t) \cos(\Omega_1 \, t) \cos(\Omega_2 \, t) \sin(\Omega_3 \, t)$

15     $S6r \propto \quad \cos(\Omega_0 \, t) \sin(\Omega_1 \, t) \cos(\Omega_2 \, t) \sin(\Omega_3 \, t)$

$S7r \propto \quad \cos(\Omega_0 \, t) \cos(\Omega_1 \, t) \sin(\Omega_2 \, t) \sin(\Omega_3 \, t)$

$S8r \propto \quad \cos(\Omega_0 \, t) \sin(\Omega_1 \, t) \sin(\Omega_2 \, t) \sin(\Omega_3 \, t).$

FT and, depending on the number n of chemical shift sine modulations the application of a zero-order phase correction of $n \cdot 90°$ yields the frequency domain spectra $A1...A8$.

20   These spectra encode Ω_1, Ω_2, and Ω_3 in signal splittings of "chemical shift multiplets" each comprising $2^K = 8$ components. Cosine and sine modulations give rise (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) to in-phase and anti-phase splittings, respectively, and linear combinations of spectra

25     $A1...A8$ provide spectra $B1...B8$ with peaks only at the frequencies of the individual multiplet components. Spectrum $B1$: $\Omega_0 + \Omega_1 + \Omega_2 + \Omega_3$, $B2$: $\Omega_0 - \Omega_1 + \Omega_2 + \Omega_3$, $B3$: $\Omega_0 + \Omega_1 - \Omega_2 + \Omega_3$, $B4$: $\Omega_0 - \Omega_1 - \Omega_2 + \Omega_3$, $B5$: $\Omega_0 + \Omega_1 + \Omega_2 - \Omega_3$, $B6$: $\Omega_0 - \Omega_1 + \Omega_2 - \Omega_3$, $B7$: $\Omega_0 + \Omega_1 - \Omega_2 - \Omega_3$, and $B8$: $\Omega_0 - \Omega_1 - \Omega_2 - \Omega_3$ (Figure 2). Spectra $B1$ to $B8$ are the "basic spectra", and the selection of chemical shift multiplet components

30   represents the phase-sensitive "editing of chemical shift multiplets".

[0106] Acquisition of peaks defining the centers of the chemical shift splittings ("central peaks") is required for unambiguous assignment, if two chemical shift quartets, $(\Omega_0, \Omega_1, \Omega_2, \Omega_3)$ and $(\Omega'_0, \Omega'_1, \Omega'_2, \Omega'_3)$, are correlated with degenerate chemical shifts in the other N_t -1 dimensions. Furthermore, degeneracy may occur between two or more shifts of the quartet itself, e.g., one may have that $\Omega_1 = \Omega'_1$. The information of the ND experiment resolving degeneracy in up to N-1 dimensions, is made available if central peaks are detected. First, spectra with transfer amplitudes of the real parts of $2^{K-1} = 4$ spectra, Sjr $(j = 9...$ 12), encode Ω_1 and Ω_2, but no Ω_3 signal splittings,

$$S9r \quad \propto \quad \cos(\Omega_0\ t)\ \cos(\Omega_1\ t)\ \cos(\Omega_2\ t)$$

$$S10r \quad \propto \quad \cos(\Omega_0\ t)\ \sin(\Omega_1\ t)\ \cos(\Omega_2\ t)$$

$$S11r \quad \propto \quad \cos(\Omega_0\ t)\ \cos(\Omega_1\ t)\ \sin(\Omega_2\ t)$$

$$S12r \quad \propto \quad \cos(\Omega_0\ t)\ \sin(\Omega_1\ t)\ \sin(\Omega_2\ t)$$

and provide the centers of the Ω_3-splittings. $S9...$ $S12$ yield, as described, spectra $B9...$ $B12$ with peaks at: $B9$: $\Omega_0 + \Omega_1 + \Omega_2$, $B10$: $\Omega_0 - \Omega_1 + \Omega_2$, $B11$: $\Omega_0 + \Omega_1 - \Omega_2$, $B12$: $\Omega_0 - \Omega_1 - \Omega_2$. Second, spectra with transfer amplitudes for the real parts $2^{K-2} = 2$ spectra, Sjr $(j = 13, 14)$, encode only Ω_1-signal splittings,

$$S13r \quad \propto \quad \cos(\Omega_0\ t)\ \cos(\Omega_1\ t)$$

$$S14r \quad \propto \quad \cos(\Omega_0\ t)\ \sin(\Omega_1\ t)$$

and provide the centers of the Ω_2-splittings. $S13$ and $S14$ yield $B13$ and $B14$ comprising peaks at: $B13$: $\Omega_0 + \Omega_1$, $B14$: $\Omega_0 - \Omega_1$. Third, $2^{K-3} = 1$ spectrum, $S15$ with a transfer amplitude for the real part encoding no signal splittings,

$$S15r \quad \propto \quad \cos(\Omega_0\ t)$$

provides the centers of the Ω_1-splittings.

[0107] GFT NMR data acquisition (Figure 1) requires recording of a total of

$$p = \sum_{k=0}^{K} 2^k = 2^{K+1} - 1\ N_tD \text{ spectra (e.g., } S1 \ ... \ S15 \text{ for } K = 3) \text{ with } 2^K \text{ basic spectra and}$$

a total of 2^{K-1} central peak spectra. This set of p data sets is designated an "(N,N_t)D

GFT NMR experiment", and central peaks due to omission of m chemical shifts are denoted to be of m-th order (e.g., $B9$... $B12$, $B13$, $B14$, and $B15$ represent first, second and third order central peaks, respectively).

[0108] For frequency domain editing, the data sets $S1...S15$ are Fourier transformed to yield spectra $A1...A15$ (Figure 2), and, depending on the number n of chemical shift sine modulations, a zero-order phase correction of $n \cdot 90°$ is applied. Subsequent linear combination yields the edited spectra $B1...B15$ (Figures 16 and 17) according to

$$\hat{B}(K) = \hat{F}(K) \cdot \hat{A}(K) \tag{4}$$

where $\hat{F}(K)$ can be readily obtained from $\hat{F}(K-1)$ by tensor product formation:

$$\hat{F}(K) = \hat{F}(K-1) \otimes \hat{F}(1) \qquad \text{with} \qquad \hat{F}(1) = \begin{bmatrix} 1 & 1 \\ 1 & -1 \end{bmatrix} \tag{5}$$

One thus obtains for $K = 2$

$$\begin{bmatrix} B9 \\ B10 \\ B11 \\ B12 \end{bmatrix} = \begin{bmatrix} 1 & 1 & 1 & 1 \\ 1 & -1 & 1 & -1 \\ 1 & 1 & -1 & -1 \\ 1 & -1 & -1 & 1 \end{bmatrix} \cdot \begin{bmatrix} A9 \\ A10 \\ A11 \\ A12 \end{bmatrix} \tag{6}$$

and for $K = 3$

$$\begin{bmatrix} B1 \\ B2 \\ B3 \\ B4 \\ B5 \\ B6 \\ B7 \\ B8 \end{bmatrix} = \begin{bmatrix} 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \\ 1 & -1 & 1 & -1 & 1 & -1 & 1 & -1 \\ 1 & 1 & -1 & -1 & 1 & 1 & -1 & -1 \\ 1 & -1 & -1 & 1 & 1 & -1 & -1 & 1 \\ 1 & 1 & 1 & 1 & -1 & -1 & -1 & -1 \\ 1 & -1 & 1 & -1 & -1 & 1 & -1 & 1 \\ 1 & 1 & -1 & -1 & -1 & -1 & 1 & 1 \\ 1 & -1 & -1 & 1 & -1 & 1 & 1 & -1 \end{bmatrix} \cdot \begin{bmatrix} A1 \\ A2 \\ A3 \\ A4 \\ A5 \\ A6 \\ A7 \\ A8 \end{bmatrix} \tag{7}$$

The equations for $K = 1$

$$\begin{bmatrix} B13 \\ B14 \end{bmatrix} = \begin{bmatrix} 1 & 1 \\ 1 & -1 \end{bmatrix} \cdot \begin{bmatrix} A13 \\ A14 \end{bmatrix} \tag{8}$$

and for $K = 0$

$$B15 = A15 \tag{9}$$

are likewise given here.

[0109]   The matrices $\hat{G}(K)$ and $\hat{F}(K)$ for time and frequency domain editing of chemical shift multiplets (Figures 1 and 2) are related to each other according to $\hat{G}(K) = \hat{H}(K) \cdot \hat{P}(K)$ with $\hat{H}(K) = \hat{F}(K) \otimes \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix}$. $\hat{H}(K)$ applies the addition scheme of $\hat{F}(K)$ (equation 5) to both real and imaginary parts. To derive $\hat{P}(K)$, $\hat{P}'(1) = \begin{bmatrix} \hat{E} & 0 \\ 0 & \hat{P} \end{bmatrix}$ is first

5   defined with $\hat{E} = \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix}$ and $\hat{P} = \begin{bmatrix} 0 & -1 \\ 1 & 0 \end{bmatrix}$. The matrix $\hat{P}$ maps the real onto the imaginary, and the imaginary onto the negative real part. This corresponds to a zero-order $90°$ phase correction in the frequency domain. Accordingly, application of $\hat{P}^n$ corresponds to applying the $n \cdot 90°$ zero-order phase correction alluded to above. $\hat{P}'(K+1)$ can be constructed from the $\hat{P}'(K)$ according to $\hat{P}'(K+1) = \hat{P}'(K) \otimes \begin{bmatrix} \hat{E} & 0 \\ 0 & \hat{P} \end{bmatrix}$.

10   Expansion of the products of $\hat{E}$ and $\hat{P}$ resulting "after" (multiple) tensor product formation yields $\hat{P}(K)$, a matrix with a 2 x 2 block diagonal form.

For $K = 3, 2,$ and 1, thus, the following is obtained for $\hat{H}(K)$ and $\hat{P}(K)$.

$$\hat{H}(3) = \begin{bmatrix} 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 \\ 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 \\ 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 \\ 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 \\ 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 \\ 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 \\ 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 \\ 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 \\ 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 & -1 \\ 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 \\ 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 \\ 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & 1 \\ 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 & -1 & 0 \\ 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 & 1 & 0 & 1 & 0 & -1 \end{bmatrix}, \quad \hat{H}(2) = \begin{bmatrix} 1 & 0 & 1 & 0 & 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 & 0 & 1 & 0 & 1 \\ 1 & 0 & -1 & 0 & 1 & 0 & -1 & 0 \\ 0 & 1 & 0 & -1 & 0 & 1 & 0 & -1 \\ 1 & 0 & 1 & 0 & -1 & 0 & -1 & 0 \\ 0 & 1 & 0 & 1 & 0 & -1 & 0 & -1 \\ 1 & 0 & -1 & 0 & -1 & 0 & 1 & 0 \\ 0 & 1 & 0 & -1 & 0 & -1 & 0 & 1 \end{bmatrix},$$

$$\hat{H}(1) = \begin{bmatrix} 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 \\ 1 & 0 & -1 & 0 \\ 0 & 1 & 0 & -1 \end{bmatrix}$$

15   and

$$\hat{P}(3) = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 \end{bmatrix}, \quad \hat{P}(2) = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 \end{bmatrix},$$

$$\hat{P}(1) = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & -1 \\ 0 & 0 & 1 & 0 \end{bmatrix}.$$

Example 2 – Options to Implement Central Peak Acquisition in GFT NMR

[0110]   The successive identification of peak pairs belonging to central peaks of *decreasing* order ensures the unambiguous assignment of chemical shift multiplet components (Figures 2 and 3). Such central peak acquisition can be achieved in three different ways. First, the *p* spectra constituting the (N,N_t)D GFT NMR experiment can be acquired by successive omission of shift evolution periods from the *N*D FT NMR radiofrequency pulse scheme affording the basic spectra (Option 1). Alternatively, central peaks can be obtained from incomplete polarization transfer (Option 2) (Szyperski et al., J. Magn. Reson. B 108:197-203 (1995), which is hereby incorporated by reference in its entirety). The exclusive use of this approach corresponds to their simultaneous acquisition in the 2^K basic spectra. Alternatively, heteronuclear steady state magnetization can be recruited (Option 3) (Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Szyperski et al., J. Biomol NMR, 11:387-405 (1998), which are hereby incorporated by reference in there entirety). For each order of central peaks obtained in such a way a recording of two subspectra is required so that the number of data sets increases twofold. The exclusive use of this approach would require recording of 4^K different N_tD data sets. These yield, after data processing, the desired $p = 2^{K+1} - 1$ spectra.

[0111]   Depending on the particular magnetization transfer pathway and practical constraints, one can combine the three options for central peak detection. The second and third option offer that (i) magnetization yielding unwanted "axial peaks" in the conventional experiment is used, (Szyperski et al., J. Am. Chem. Soc.

118:8146-8147 (1996), which is hereby incorporated by reference in its entirety) and that (ii) central peaks are registered even if the resonances in the higher-order spectra are broadened. Overall, $2^K < p < 4^K$ data sets thus need to be recorded to obtain the (N,N_t)D GFT NMR experiment: the resulting "sampling demand" is equivalent to recording an ND FT NMR experiment with only "one (2^K data sets) to two (4^K data sets) complex points" in each of the K dimensions.

Example 3 – Formula to Calculate the Reductions in Minimal Measurement Times for Different Options for Central Peak Acquisition

[0112] With respect to Option 1 for central peak acquisition (see Example 2), if the p data sets defining the (N,N_t)D experiment are separately recorded, the ratio ε_1 is as defined in equation 3:

$$\varepsilon_1 = \frac{T_m(FT)}{T_m(GFT)} = (2^K/(2^{K+1}-1)) \cdot \left(\prod_{j=0}^{K} n_j\right) / \left(\sum_{j=0}^{K} n_j\right), \qquad (3), (10)$$

[0113] With respect to Option 2 for central peak acquisition (see Example 2), if the basic spectra are recorded with simultaneous acquisition of central peaks from incomplete INEPT, one obtains ε_2:

$$\varepsilon_2 = \frac{T_m(FT)}{T_m(GFT)} = \left(2^{K+1} \cdot \prod_{j=0}^{K} n_j\right) / \left(2^K \cdot 2 \cdot \sum_{j=0}^{K} n_j\right) = \left(\prod_{j=0}^{K} n_j\right) / \left(\sum_{j=0}^{K} n_j\right), \qquad (11),$$

i.e., the ratio becomes simply the product of the number of points over the corresponding sum.

[0114] With respect to Option 3 for central peak acquisition (see Example 2), if heteronuclear magnetization is exclusively used for central peak detection, one obtains ε_3:

$$\varepsilon_3 = \frac{T_m(FT)}{T_m(GFT)} = (1/2^K) \cdot \left(\prod_{j=0}^{K} n_j\right) / \left(\sum_{j=0}^{K} n_j\right). \qquad (12).$$

[0115] Table 1 illustrates the representative calculations of the reductions in minimal measurement times in GFT NMR.

Table 1. The Reduction of Minimal Measurement Times, ε, for $K = 1, 2, 3$ and Different Approaches for Central Peak Detection Assuming That Each of the Projected $K+1$ Dimensions are Sampled with 16(32) Complex Points

	2^K basic data sets[a] equation 11	$2^{K+1}-1$ data sets[b] equation 3	4^K data sets[c] equation 12
$K = 1$	$\varepsilon_2 = 8\ (16)$	$\varepsilon_1 = 5.3\ (10.7)$	$\varepsilon_3 = 4\ (8)$
$K = 2$	$\varepsilon_2 = 85\ (341)$	$\varepsilon_1 = 48.6\ (195)$	$\varepsilon_3 = 21\ (85)$
$K = 3$	$\varepsilon_2 = 1024\ (8192)$	$\varepsilon_1 = 546\ (4369)$	$\varepsilon_3 = 128\ (1024)$

[a] Central peaks obtained exclusively from incomplete polarization transfer (Szyperski et al., J. Magn. Reson. B 108:197-203 (1995), which is hereby incorporated by reference in its entirety).

[b] Central peaks obtained by successive omission of chemical shift evolution periods in the parent experiment.

[c] Central peaks exclusively obtained from heteronuclear steady-state magnetization (Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996), which is hereby incorporated by reference in its entirety).

[0116]   For the implementation of (5,2)D HACACONHN, $^{13}C^\alpha$ steady state magnetization was used to detect the first order central peaks defining the $\Omega_1(^1H^\alpha)$ splittings (see Examples 4 and 5), which yields a second set of 8 data sets. Second and third order central peaks defining, respectively, the $\Omega_2(^{13}C^\alpha)$ and $\Omega_1(^{13}C')$ splittings were obtained from separate recording of somewhat higher resolved reduced-dimensionality (Szyperski et al., Proc. Natl. Acad. Sci. USA, 99:8009-8014 (2002); Szyperski et al. J. Biomol. NMR. 3:127–132 (1993); Szyperski et al., J. Am. Chem. Soc. 115:9307-9308 (1993); Szyperski et al., J. Magn. Reson. B 105:188-191 (1994); Brutscher et al., J. Magn. Reson. B 105:77-82 (1994); Szyperski et al., J. Magn. Reson. B 108:197-203 (1995); Brutscher et al., J. Magn. Reson. B 109:238-242 (1995); Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Bracken et al., J. Biomol. NMR 9:94-100 (1997); Szyperski et al., J. Biomol NMR, 11:387-405 (1998); Astrof et al., J. Magn. Reson. 152:303-307 (2001); Xia et al., J. Biomol. NMR 24:41-40 (2002), which are hereby incorporated by reference in their entirety). 2D HNNCO, an experiment derived from the HNNCO scheme (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996), which is hereby incorporated

by reference in its entirety) (two data sets), and 2D [^{15}N,^{1}H]-HSQC (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety), respectively (Figure 16). The use of ^{13}C$^\alpha$ steady-state magnetization for first order central peak detection yields ε = 250 being intermediate between ε$_1$ (equation 3) and ε$_3$ (equation 12).

Example 4 - NMR Spectroscopy

[0117]   For the 76-residue protein ubiquitin nearly all signals of 2D [^{15}N,^{1}H]-HSQC (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) are resolved so that $N_t = 2$ is an obvious choice. As an application, a (5,2)D HACACONHN GFT NMR experiment ($K = 3$) was, thus, recorded within 138 minutes on a VARIAN INOVA 600 spectrometer using the HACACONHN rf pulse sequence shown in Figure 6. This experiment correlates the polypeptide backbone ^{1}H$^\alpha$, ^{13}C$^\alpha$ and ^{13}C' chemical shifts of residue i with the backbone amide ^{15}N and ^{1}H^N chemical shifts of residue $i+1$. The underlined letters denote that $\Omega_3(^1$H$^\alpha)$, $\Omega_2(^{13}$C$^\alpha)$, $\Omega_1(^{13}$C') and $\Omega_0(^{15}$N) are measured in a single dimension. A 2 mM solution of ^{15}N/^{13}C doubly labeled ubiquitin in 90% H$_2$O/10% D$_2$O (50 mM K-PO$_4$; pH = 5.8) was used at T = 25°C.

[0118]   With the HACACONHN rf pulse scheme of Figure 6, sixteen individual data sets R1-R16 (to provide basic and first-order central peaks) were acquired in 6.9 minutes each, with SW$_1$(^{15}N/^{13}C'/^{13}C$^\alpha$/^{1}H$^\alpha$) = 8,000 Hz and 53(t_1)*512(t_2) complex points [$t_{1,max}$(^{15}N/^{13}C'/^{13}C$^\alpha$/^{1}H$^\alpha$) = 6.5 ms; $t_{2,max}$(^{1}H^N) = 73.2 ms], yielding after data processing (see Example 5) the twelve planes B1-B12 containing basic and first-order central peaks. The phase of the 90° rf pulses generating transverse ^{15}N, ^{13}C', ^{13}C$^\alpha$ and ^{1}H$^\alpha$ magnetization for frequency labeling are ϕ_0, ϕ_1, ϕ_2, and ϕ_3, respectively (Figure 6). ϕ_0 is altered between 0° and 90° for phase sensitive acquisition (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) of $\Omega_0(^{15}$N) along t_1. The three phases ϕ_1, ϕ_2 and ϕ_3 are

independently altered between $0°$ and $90°$ for frequency discrimination of $\Omega_1(^{13}C')$, $\Omega_2(^{13}C^\alpha)$, and $\Omega_3(^1H^\alpha)$ in the first eight data sets $R1$-$R8$. For first order central peak detection using ^{13}C steady-state magnetization (Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Szyperski et al., J. Biomol NMR, 11:387-405 (1998), which

5   are hereby incorporated by reference in their entirety), the eight measurements are repeated with the first $90°$ pulse on $^1H^\alpha$ being shifted by $180°$. This yields the following phase cycle for the 16 data sets $R1$-$R16$: $\phi_1 = 8(x, y)$; $\phi_2 = 4(2x, 2y)$; $\phi_3 = 4x$, $4y$, $4(-x)$, $4(-y)$ with the receiver phase being unchanged. A reduced dimensionality (Szyperski et al., Proc. Natl. Acad. Sci. USA, 99:8009-8014 (2002); Szyperski et al.,

10   J. Am. Chem. Soc. 115:9307-9308 (1993), which are hereby incorporated by reference in their entirety) 2D HNNCO spectrum (second order central peaks) derived from a HNNCO scheme (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety) was acquired in 13.8 minutes with $SW(^{15}N/^{13}C') = 8,000$ Hz and $128(t_1)*512(t_2)$

15   complex points $[t_{1,max}(^{15}N/^{13}C') = 15.9$ ms; $t_{2,max}(^1H^N) = 73.2$ ms], yielding data sets $R17$ and $R18$ ($B13$ and $B14$ after data processing; see Example 5) with phase $\phi_3 = (x, y)$. A 2D $[^{15}N,^1H]$-HSQC spectrum (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) (third order central peaks) was acquired in

20   13.8 minutes with $SW(^{15}N) = 8,000$ Hz, and $256(t_1)*512(t_2)$ complex points $[t_{1,max}(^{15}N) = 26$ ms; $t_{2,max}(^1H^N) = 73.2$ ms], yielding the data set $R19$ ($B15$ after data processing; see Example 5). For larger systems requiring longer measurements, it might be advisable to derive second and third order central peaks from $^{13}C'$ and ^{15}N steady state magnetization, respectively. The total measurement time of the 19 data

25   sets was 138 minutes. To obtain pure phases, zero first-order phase corrections must be ensured along ω_1 by, for example, starting sampling at $t_1 = 0$ for all of the combined chemical shift evolution periods. Editing of chemical shift multiplets in the time domain is advantageous, because the extension of the time domain data by linear prediction (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two

30   Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety) (from 53 to 106 complex points for data sets $T1$ to $T12$ and from 128 to 192 for data sets $T13$ and $T14$) profits from both maximizing the signal-to-noise of

the time domain data and reducing the number of chemical shifts ("oscillators") to be predicted. The digital resolution after FT and zero-filling was 7.8 Hz/point along ω_1 and 6.9 Hz/point along ω_2.

[0119] A 5D FT HACACONHN spectrum acquired with the same maximal evolution times as the basic spectra of (5,2)D HACACONHN would require sampling of $10(t_1)*11(t_2)*22(t_3)*13(t_4)*512(t_5)$ complex points [i.e., $n = \left(\sum_{j=0}^{K} n_j\right) - K$] with spectral widths of $SW_1(^{15}N) = 1{,}440$ Hz, $SW_2(^{13}C') = 1{,}500$ Hz, $SW_3(^{13}C^\alpha) = 3{,}260$ Hz, and $SW_4(^1H^\alpha) = 1{,}800$ Hz (i.e., $SW = \sum_{j=0}^{K} SW_j$) in 5.83 days of spectrometer time.

For comparison of digital resolution in FT and GFT NMR, 2D $[\omega_1, \omega_5]$-, $[\omega_2, \omega_5]$-, $[\omega_3, \omega_5]$- and $[\omega_4, \omega_5]$-planes of the 5D FT HACACONHN experiment were recorded in 1.3, 1.4, 2.9 and 1.7 minutes, respectively. For line width comparisons with (5,2)D GFT HACACONHN, the same planes were also acquired with spectral widths of SW = 8,000 Hz in the indirect dimension.

Example 5 – Data Processing of the (5,2)D HACACONHN GFT NMR Spectrum

[0120] First order central peaks were derived from ^{13}C steady state magnetization. (Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Szyperski et al., J. Biomol NMR, 11:387-405 (1998), which are hereby incorporated by reference in their entirety). This requires a "pre-processing" prior to G-matrix transformation. The data sets $R1$-$R16$ are combined to yield the basic data sets, $S1...$ $S8$, and first order central peak data sets, $S9...$ $S12$, respectively, according to:

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \\ S7 \\ S8 \end{bmatrix} = \begin{bmatrix} 1\,0\,0\,0\,0\,0\,0\,0\,-1\,0\,\;0\,\;0\,\;0\,\;0\,\;0\,\;0 \\ 0\,1\,0\,0\,0\,0\,0\,0\,\;0\,-1\,0\,\;0\,\;0\,\;0\,\;0\,\;0 \\ 0\,0\,1\,0\,0\,0\,0\,0\,\;0\,\;0\,-1\,0\,\;0\,\;0\,\;0\,\;0 \\ 0\,0\,0\,1\,0\,0\,0\,0\,\;0\,\;0\,\;0\,-1\,0\,\;0\,\;0\,\;0 \\ 0\,0\,0\,0\,1\,0\,0\,0\,\;0\,\;0\,\;0\,\;0\,-1\,0\,\;0\,\;0 \\ 0\,0\,0\,0\,0\,1\,0\,0\,\;0\,\;0\,\;0\,\;0\,\;0\,-1\,0\,\;0 \\ 0\,0\,0\,0\,0\,0\,1\,0\,\;0\,\;0\,\;0\,\;0\,\;0\,\;0\,-1\,0 \\ 0\,0\,0\,0\,0\,0\,0\,1\,\;0\,\;0\,\;0\,\;0\,\;0\,\;0\,\;0\,-1 \end{bmatrix} \cdot [R1 \ldots R8\,R9 \ldots R16]^T \quad (13).$$

and

$$\begin{bmatrix} S9 \\ S10 \\ S11 \\ S12 \end{bmatrix} = \begin{bmatrix} 0\,0\,1\,0\,0\,0\,1\,0\,0\,0\,1\,0\,0\,0\,1\,0 \\ 0\,0\,0\,1\,0\,0\,0\,1\,0\,0\,0\,1\,0\,0\,0\,1 \\ 1\,0\,0\,0\,1\,0\,0\,0\,1\,0\,0\,0\,1\,0\,0\,0 \\ 0\,1\,0\,0\,0\,1\,0\,0\,0\,1\,0\,0\,0\,1\,0\,0 \end{bmatrix} \cdot [R1 \ldots R8\,R9 \ldots R16]^T \quad (14).$$

This corresponds to the difference and sum formation for central peak acquisition using $^{13}C^{\alpha}$ steady state magnetization (Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Szyperski et al., J. Biomol NMR, 11:387-405 (1998), which are hereby

5 incorporated by reference in their entirety). Transverse ^{13}C magnetization originating from ^{13}C longitudinal steady state magnetization is 90° out of phase relative to transverse ^{13}C magnetization originating from ^{1}H magnetization. (Szyperski et al., J. Am. Chem. Soc. 118:8146-8147 (1996); Szyperski et al., J. Biomol NMR, 11:387-405 (1998), which are hereby incorporated by reference in their entirety). Hence,

10 $\sin[\Omega(^{13}C^{\alpha})t]$ and $\cos[\Omega(^{13}C^{\alpha})t]$ modulation are "swapped" for central peak acquisition and rows 1 and 2, and 3 and 4 in equation 14 are exchanged compared to equation 13. With $S13$ and $S14$ from 2D HNNCO, and $S15$ from 2D [$^{15}N,^{1}H$]-HSQC, the $p = 2^{K+1} - 1 = 15$ data sets constituting the (5,2)D HACACONHN experiment become available. The required phase sensitive editing of the chemical shift multiplet

15 components can be achieved either in the frequency (see Example 1) or the time domain (equation 1).

[0121] $\hat{G}_{c}(K)$, as defined by equation 1, can be decomposed into real and imaginary part, $\hat{G}_{c}(K) = \hat{R}(K) + i \cdot \hat{I}(K)$. With $\hat{R}_{j}(K)$ and $\hat{I}_{j}(K)$ denoting the corresponding j-th row vectors, one then obtains the real $2^{K+1} \times 2^{K+1}$ square G-matrix,

20 $\hat{G}(K) = [\hat{R}_{1} \ \hat{I}_{1} \ \hat{R}_{2} \ \hat{I}_{2} \ ... \ \hat{R}_{2^{K}} \ \hat{I}_{2^{K}}]^{r}$, which transforms $\hat{S}(K)$ into

$\hat{T}(K) = [T1r \ T1i \ T2r \ T2i \ ... \ T2^{K}r \ T2^{K}i]^{r}$ according to $\hat{T}(K) = \hat{G}(K) \cdot \hat{S}(K)$. For time domain editing of the (5,2)D HACACONHN experiment, one thus obtains the following real G-matrices for $K = 3$ (basic spectra):

$$
\begin{bmatrix} T1r \\ T1i \\ T2r \\ T2i \\ T3r \\ T3i \\ T4r \\ T4i \\ T5r \\ T5i \\ T6r \\ T6i \\ T7r \\ T7i \\ T8r \\ T8i \end{bmatrix} =
\begin{bmatrix}
1 & 0 & 0 & -1 & 0 & -1 & -1 & 0 & 0 & -1 & -1 & 0 & -1 & 0 & 0 & 1 \\
0 & 1 & 1 & 0 & 1 & 0 & 0 & -1 & 1 & 0 & 0 & -1 & 0 & -1 & -1 & 0 \\
1 & 0 & 0 & 1 & 0 & -1 & 1 & 0 & 0 & -1 & 1 & 0 & -1 & 0 & 0 & -1 \\
0 & 1 & -1 & 0 & 1 & 0 & 0 & 1 & 1 & 0 & 0 & 1 & 0 & -1 & 1 & 0 \\
1 & 0 & 0 & -1 & 0 & 1 & 1 & 0 & 0 & -1 & -1 & 0 & 1 & 0 & 0 & -1 \\
0 & 1 & 1 & 0 & -1 & 0 & 0 & 1 & 1 & 0 & 0 & -1 & 0 & 1 & 1 & 0 \\
1 & 0 & 0 & 1 & 0 & 1 & -1 & 0 & 0 & -1 & 1 & 0 & 1 & 0 & 0 & 1 \\
0 & 1 & -1 & 0 & -1 & 0 & 0 & -1 & 1 & 0 & 0 & 1 & 0 & 1 & -1 & 0 \\
1 & 0 & 0 & -1 & 0 & -1 & -1 & 0 & 0 & 1 & 1 & 0 & 1 & 0 & 0 & -1 \\
0 & 1 & 1 & 0 & 1 & 0 & 0 & -1 & -1 & 0 & 0 & 1 & 0 & 1 & 1 & 0 \\
1 & 0 & 0 & 1 & 0 & -1 & 1 & 0 & 0 & 1 & -1 & 0 & 1 & 0 & 0 & 1 \\
0 & 1 & -1 & 0 & 1 & 0 & 0 & 1 & -1 & 0 & 0 & -1 & 0 & 1 & -1 & 0 \\
1 & 0 & 0 & -1 & 0 & 1 & 1 & 0 & 0 & 1 & 1 & 0 & -1 & 0 & 0 & 1 \\
0 & 1 & 1 & 0 & -1 & 0 & 0 & 1 & -1 & 0 & 0 & 1 & 0 & -1 & -1 & 0 \\
1 & 0 & 0 & 1 & 0 & 1 & -1 & 0 & 0 & 1 & -1 & 0 & -1 & 0 & 0 & -1 \\
0 & 1 & -1 & 0 & -1 & 0 & 0 & -1 & -1 & 0 & 0 & -1 & 0 & -1 & 1 & 0
\end{bmatrix}
\begin{bmatrix} S1r \\ S1i \\ S2r \\ S2i \\ S3r \\ S3i \\ S4r \\ S4i \\ S5r \\ S5i \\ S6r \\ S6i \\ S7r \\ S7i \\ S8r \\ S8i \end{bmatrix}
$$

(15),

for $K = 2$ (first order central peaks):

$$
\begin{bmatrix} T9r \\ T9i \\ T10r \\ T10i \\ T11r \\ T11i \\ T12r \\ T12i \end{bmatrix} =
\begin{bmatrix}
1 & 0 & 0 & -1 & 0 & -1 & -1 & 0 \\
0 & 1 & 1 & 0 & 1 & 0 & 0 & -1 \\
1 & 0 & 0 & 1 & 0 & -1 & 1 & 0 \\
0 & 1 & -1 & 0 & 1 & 0 & 0 & 1 \\
1 & 0 & 0 & -1 & 0 & 1 & 1 & 0 \\
0 & 1 & 1 & 0 & -1 & 0 & 0 & 1 \\
1 & 0 & 0 & 1 & 0 & 1 & -1 & 0 \\
0 & 1 & -1 & 0 & -1 & 0 & 0 & -1
\end{bmatrix} \cdot
\begin{bmatrix} S9r \\ S9i \\ S10r \\ S10i \\ S11r \\ S11i \\ S12r \\ S12i \end{bmatrix}
$$

(16),

for $K = 1$ (second order central peaks):

$$
\begin{bmatrix} T13r \\ T13i \\ T14r \\ T14i \end{bmatrix} =
\begin{bmatrix}
1 & 0 & 0 & -1 \\
0 & 1 & 1 & 0 \\
1 & 0 & 0 & 1 \\
0 & 1 & -1 & 0
\end{bmatrix} \cdot
\begin{bmatrix} S13r \\ S13i \\ S14r \\ S14i \end{bmatrix}
$$

(17),

and for $K = 0$ (third order central peaks):

$$
\begin{bmatrix} T15r \\ T14i \end{bmatrix} =
\begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix} \cdot
\begin{bmatrix} S15r \\ S15i \end{bmatrix}
$$

(18).

Since real and imaginary parts are recorded for all four chemical shifts Ω_0, Ω_1, Ω_2, and Ω_3 in the basic spectra (equation 15), the particular choice of Ω_0 is, in principle, arbitrary. A suitable rearrangement of the FIDs allows one to exchange Ω_0 with any

of the other three chemical shifts after data acquisition. However, the order chosen for central peak detection defines Ω_0. For the (5,2)D HACACONHN experiment, 2D $[^{15}N,^{1}H]$-HSQC is the most sensitive choice for third order central peak detection so that $\Omega_0 = \Omega\,(^{15}N)$.

Example 6 – Peak Assignment and Calculation of Chemical Shifts

[0122]   The chemical shift multiplets encoded in the edited spectra $B1...B15$ of (5,2)D HACACONHN were identified starting from an assigned $[^{15}N,^{1}H]$-HSQC peak list in the "bottom-up" manner described in Figure 3. The resulting peak lists of $B1...B15$ were then used as input for a least squares fitting routine (Eadie et al., Statistical Methods in Experimental Physics, North-Holland, New York (1982); Lau, A Numerical Library in C for Scientists and Engineers, CRC Press, Boca Raton (1995), which are hereby incorporated by reference in their entirety) solving an overdetermined system of 15 equations resulting from the ω_1-frequencies of the 15 peaks. This yielded the correlations involving $\Omega(^{1}H^{\alpha}_i)$, $\Omega(^{13}C^{\alpha}_i)$, $\Omega\,(^{13}C'_i)$, $\Omega(^{15}N_{i+1})$, and $\Omega(^{1}H^{N}_{i+1})$ (Table 2). A Monte Carlo simulation of error propagation (see description of Figure 19 for details) served to provide an estimate for the standard deviations for the chemical shift measurements based on the measurements of line widths.

Table 2. Chemical Shifts[a] of Ubiquitin Measured in (5,2)D HACACONHN. The Following Standard Deviations Were Obtained (Figures 16-19); $\sigma(^{15}N) = \pm$ 0.043 ppm (2.4 Hz), $\sigma(^1H^N)^b = \pm$ 0.006 ppm (3.3 Hz), $\sigma(^{13}C') = \pm$ 0.017 ppm (2.6 Hz), $\sigma(^{13}C^\alpha) = \pm$ 0.019 ppm (2.9 Hz), $\sigma(^1H^\alpha) = \pm$ 0.006 ppm (3.7 Hz).

Residue	$\delta(^{15}N)$	$\delta(^1H^N)^a$	$\delta(^{13}C')$	$\delta(^{13}C^\alpha)$	$\delta(^1H^\alpha)$
M1			170.500	54.505	4.220
Q2	122.911	8.965	176.021	55.178	5.289
I3	115.095	8.328	172.351	59.678	4.161
F4	118.498	8.624	175.151	55.162	5.642
V5	121.256	9.317	174.775	60.430	4.815
K6	127.847	8.954	177.095	54.682	5.293
T7	115.413	8.762	176.890	60.564	4.932
L8	121.248	9.129	178.821	57.561	4.302
T9	105.850	7.656	175.494	61.463	4.423
G10	109.192	7.845	173.952	45.428	3.612/4.344
K11	121.890	7.286	175.722	56.318	4.359
T12	120.604	8.657	174.334	62.380	5.051
I13	127.688	9.562	175.149	60.029	4.522
T14	121.650	8.757	173.731	62.043	4.977
L15	125.150	8.751	174.525	52.827	4.754
E16	122.459	8.139	175.802	54.968	4.889
V17	117.511	8.956	174.039	58.479	4.694
E18	119.306	8.679			
P19			175.261	65.330	4.124
S20	103.394	7.048	174.592	57.431	4.360
D21	123.869	8.070	176.285	55.924	4.687
T22	108.971	7.895	176.760	59.658	4.905
I23	121.290	8.538			
E24[c]			178.964	60.734	3.890
N25	121.406	7.942	178.288	56.068	4.558
V26	122.156	8.118	177.918	67.684	3.398
K27	118.967	8.580	180.483	59.222	4.592
A28	123.484	7.994	180.214	55.419	4.161
K29	120.211	7.875	180.257	59.793	4.202
I30	121.352	8.301	178.151	66.124	3.487

Table 2 (continued)

Residue	δ(^{15}N)	δ(^{1}H^N)[a]	δ(^{13}C')	δ(^{13}C$^\alpha$)	δ(^{1}H$^\alpha$)
Q31	123.551	8.568	178.820	60.067	3.822
D32	119.733	8.036	177.278	57.454	4.333
K33	115.432	7.446	177.777	58.193	4.310
E34	114.269	8.742	177.909	55.395	4.570
G35	108.796	8.518	173.915	46.100	3.929/4.135
I36	120.298	6.174			
P38			178.246	66.138	4.116
D39	113.588	8.546	177.032	55.819	4.411
Q40	116.885	7.834	175.381	55.647	4.463
Q41	118.029	7.498	176.133	56.684	4.223
R42	123.033	8.524	173.854	55.186	4.483
L43	124.390	8.843	175.257	53.051	5.367
I44	122.295	9.119	175.795	58.972	4.943
F45	125.055	8.866	174.578	56.597	5.161
A46	132.938	8.993	177.317	52.598	3.697
G47	102.422	8.138	173.732	45.412	3.450/4.100
K48	121.961	7.999	174.627	54.640	4.598
Q49	123.000	8.664	175.566	55.922	4.540
L50	125.679	8.579	176.611	54.287	4.090
E51	123.110	8.407	175.475	55.979	4.511
D52	120.354	8.179			
G53[c]			174.754	45.245	4.062
R54	119.329	7.482	175.316	54.338	4.725
T55	108.815	8.847	176.490	59.700	5.237
L56	118.016	8.168	180.752	58.673	4.053
S57	113.484	8.499	178.282	61.149	4.242
D58	124.505	7.954	177.401	57.440	4.291
Y59	115.770	7.276	174.663	58.295	4.651
N60	115.940	8.174	174.256	54.179	4.351
I61	118.831	7.264	174.514	62.470	3.371
Q62	124.948	7.642	175.751	53.653	4.477
K63	120.514	8.505	175.694	57.905	3.979
E64	114.574	9.335	175.205	58.419	3.330

Table 2 (continued)

Residue	$\delta(^{15}N)$	$\delta(^1H^N)^a$	$\delta(^{13}C')$	$\delta(^{13}C^\alpha)$	$\delta(^1H^\alpha)$
S65	114.914	7.683	172.008	60.931	4.632
T66	117.439	8.742	173.741	62.506	5.291
L67	127.691	9.432	175.314	53.866	5.085
H68	119.271	9.255	173.703	56.002	5.141
L69	123.921	8.311	175.356	53.848	5.184
V70	126.737	9.202	174.033	60.654	4.378
L71	123.087	8.125	177.806	54.030	5.021
R72	123.792	8.620	175.284	55.713	4.262
L73	124.533	8.372	177.388	54.846	4.396
R74	121.936	8.453	176.837	56.613	4.303
G75	111.089	8.505	173.633	45.331	3.966
G76	115.040	7.959			

[a] Chemical shifts are in ppm and relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).
[b] Directly measured chemical shift.
[c] Glu 24 and Gly 53 show broadened ^{15}N-$^1H^N$ resonances.

Example 7 – Analysis of the (5,2)D HACACONHN GFT NMR Experiment

[0123]   A (5,2)D HACACONHN GFT NMR experiment for the 8.6 kDa
protein ubiquitin was acquired as an application of the GFT NMR spectroscopy.
Figure 16 shows the chemical shift multiplets as well as the resulting edited multiplet
components, and Figure 17 shows all 15 planes constituting the (5,2)D
HACACONHN experiment. The bottom up identification (Figure 3) of components
forming a given shift multiplet allows one to retain the 5D correlations of the parent
experiment. Peak detection was nearly complete so that a total of 67 chemical shift 5-
tuples as well as 3 shift 6-tuples for glycines with non-degenerate $^1H^\alpha$ shifts (Table 2)
were obtained. The S/N ratios obtained in the GFT NMR experiment (The S/N ratios
were between 6.4 and 12.0 in the basic spectra (Figure 17A), and between 5.6 and
10.4 for first-order peaks (Figure 17B), between 9.8 and 24.0 for second-order peaks
(Figure 17C), and between 44.0 and 108.0 for third-order central peaks (Figure 17D).)
demonstrate adequate adjustment of the measurement time to sensitivity requirements
while the desired 5D chemical shift correlations were registered. The ratios also show

that conventional 4D or 5D HACACONHN experiments had to be acquired in the sampling limited data collection regime because their minimal measurements are in the order of several days.

[0124]   Because equivalent chemical shift correlations are provided by (5,2)D HACACONHN GFT and 5D HACACONHN FT NMR, these two experiments can be compared in terms of minimal measurement times and data sizes. An evident advantage of the GFT NMR experiment is the large reduction in T_m. Equation 3 predicts reductions in measurement times of about an order of magnitude for each dimension included into the joint sampling scheme (Table 1). In fact, the minimal measurement times with a single scan per FID each second (and the same t_{max} for all chemical shift evolution periods as chosen for basic and first order central peak acquisition) are 33.5 min. and 5.83 days for (5,2)D HACACONHN GFT NMR (Figure 17) and 5D HACACONHN FT NMR, revealing a 250-fold reduction in T_m for the GFT experiment (note that this value deviates from $\varepsilon = 317$ obtained with equation 2, due to the particular choice to implement central peak acquisition; see Example 3). Concomitantly, the data size is largely reduced when transformed data sets with equal digital resolution are compared (Figure 18).

[0125]   In order to assess the precision of the chemical shift measurements the resonance line widths need to be considered (Ernst et al., Principles of Nuclear Magnetic Resonance in One and Two Dimensions, Clarendon, Oxford (1987), which is hereby incorporated by reference in its entirety). In general, the joint sampling of K + 1 "non constant-time" chemical shift evolution periods yields transfer amplitudes attenuated by $\exp\left(-\sum_{j=0}^{K} R_{2,j} \cdot t\right)$, where $R_{2,j}$ represents the transverse relaxation rate constant of the j-th dimension. However, higher-dimensional heteronuclear FT NMR shift correlation spectra are quite often recorded with frequency labeling being accomplished in a constant-time manner (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety) and/or with $t_{j,max} \ll 1/R_{2,j}$ for all j. As for the implementation of (5,2)D HACACONHN, the linewidth is then determined by the t_{max} values but is not dependent on $R_{2,j}$. Assuming for simplicity that all $t_{j,max}$ are identical, the 2^K lines of the chemical shift multiplets exhibit the same width as the

corresponding single peak in ND FT NMR along each of the dimensions. Hence, peaks are not broadened in constant-time GFT NMR spectra with increasing K (The width at half height of the frequency domain sinc centre lobe resulting from truncation in the time domain at t_{max} is given[2] by $0.604/t_{max}$. In the current implementation of (5,2)D HACACONHN (Figure 6) all indirect evolution periods except for $\Omega(^1H^\alpha)$ are constant time periods. The evolution of Ω $(^1H^\alpha)$ is implemented in a semi constant time manner (Cavanagh et al., Protein NMR Spectroscopy, Academic Press, San Diego (1996), which is hereby incorporated by reference in its entirety), so that signal losses due to transverse relaxation of $^1H^\alpha$ are negligible for 8.6 kDa ubiquitin at short t_{max} values around 6.5 ms. For larger systems with short $T_2(^1H^\alpha)$, however, the semi constant time frequency labeling may lead to a detectable increase of ω_1-linewidths in the basic when compared to central peak spectra.) This is neatly confirmed when comparing ω_1 cross sections from (5,2)D HACACONHN with those taken from 2D HACACONHN spectra (Figures 18A-B).

[0126] The fact that the individual multiplet components possess the same line widths as the corresponding signals in the parent FT NMR experiment (Figures 18A-B) has a profound impact on the precision of the chemical shift measurement in constant time GFT NMR experiments such as (5,2)D HACACONHN. To relate line widths to errors of measurement, a conservative statistical model was adopted in which (i) the error for the identification of peak positions is associated with a Gaussian distribution and (ii) the Lorenzian line width, $\Delta\nu_{1/2}$, represents the corresponding 99.5% confidence interval (i.e., $\Delta\nu_{1/2} = 6\sigma$). σ (basic), σ (first), σ (second) and σ (third) are the standard deviations for shift measurements in basic, first order, second order and third order central peak spectra, respectively. Considering (i) that lines do not broaden with increasing K (Figures 18A-B) and (ii) the different maximal evolution times (see Example 4), one has that σ (basic) = σ (first) = $\sigma^{FT}(^{13}C^\alpha) = \sigma^{FT}(^1H^\alpha)$, σ (second) = $\sigma^{FT}(^{13}C')$ and σ (third) = $\sigma^{FT}(^{15}N)$. $\sigma^{FT}(X)$ represents the standard deviation for the chemical shift measurement of nucleus X $(^1H^\alpha, {}^{13}C^\alpha, {}^{13}C', {}^{15}N)$ in conventional FT NMR spectra acquired with corresponding t_{max}. Monte Carlo simulations were performed to calculate the standard deviations $\sigma(^{13}C^\alpha)$, $\sigma(^1H^\alpha)$, $\sigma(^{13}C')$, and $\sigma(^{15}N)$ in (5,2)D HACACONHN GFT NMR for various selections of subspectra (Figures 19-20). If a minimal number of four basic

spectra is selected to calculate $\Omega_3(^1H^\alpha)$, $\Omega_2(^{13}C^\alpha)$, $\sigma_1(^{13}C')$ and $\Omega_0(^{15}N)$, the precision depends on which four are selected (see Figure 20 and its description for details). In the two most favorable cases, the standard deviations in the constant time GFT NMR experiment are reduced by a factor of $2 = \sqrt{4}$, that is, $\sigma(X) = \frac{1}{2} \cdot \sigma^{FT}(^1H^\alpha) =$

5    $\frac{1}{2} \cdot \sigma^{FT}(^{13}C^\alpha) = \frac{1}{2} \cdot \sigma(\text{basic})$. If the 8 basic spectra are selected, the standard deviation is

reduced by an additional factor of $\sqrt{2}$, yielding $\sigma(X) = \frac{1}{\sqrt{8}} \cdot \sigma$ (basic). Similarly,

$\sigma(X) = \frac{1}{\sqrt{12}} \cdot \sigma$ (basic) if both the 8 basic and 4 first order central peak spectra are

chosen. The exact match between reductions by a factor of $\sqrt{n}$, where n represents the number of spectra, and the reductions obtained from the simulations (see

10   descriptions of Figures 19-20 for details) reflects the well-known relation from statistics (Eadie et al., Statistical Methods in Experimental Physics, North-Holland, New York (1982), which is hereby incorporated by reference in its entirety) stating that the standard deviation of an average arising from n multiple independent measurements is reduced by a factor of $\sqrt{n}$ (Figure 1). For the implementation of

15   (5,2)D HACACONHN, second- and third-order central peak spectra were acquired with longer maximal evolution times than the first-order central peak and basic spectra (Figure 17E; see Example 4). Hence, $\sigma(^{13}C')$ and $\sigma(^{15}N)$ turned out to be somewhat smaller than $\frac{1}{\sqrt{14}} \cdot \sigma(\text{basic})$ and $\frac{1}{\sqrt{15}} \cdot \sigma(\text{basic})$, respectively, when 14 or all

15 spectra are considered (see description of Figure 20 for details). When compared

20   with $\sigma(\text{second}) = \sigma^{FT}(^{13}C')$ and $\sigma(\text{third}) = \sigma^{FT}(^{15}N)$, which reflect rather long maximal evolution times, the values of $\sigma(^{13}C')$ and $\sigma(^{15}N)$ are reduced by factors of 2.5 and 2.0, respectively. The Monte Carlo simulations are in neat agreement with analytical calculations of standard deviations using the Gaussian law of error propagation (Eadie et al., Statistical Methods in Experimental Physics, North-Holland, New York (1982),

25   which is hereby incorporated by reference in its entirety) (see description of Figure 20) and are evidently a valuable tool to analyze the precision of shift measurements in more intricate future implementations of GFT NMR experiments.

[0127]   Overall, the precision of the indirect shift measurements in the (5,2)D HACACONHN experiment [$\sigma(^1H^\alpha)$ = 3.7 Hz, $\sigma(^{13}C^\alpha)$ = 2.9 Hz, $\sigma(^{13}C')$ = 2.6 Hz, $\sigma(^{15}N)$ = 2.4 Hz] matched the one obtained in the direct dimension [$\sigma(^1H^N)$ = 3.3 Hz]. Remarkably, one can anticipate for molecules tumbling slower than ubiquitin at 25°C, that the precision of the indirectly detected shifts will be higher than for the directly detected amide proton shift. This is because the precision of shift measurements in the indirect constant-time evolution periods is determined by t_{max} (which would not change for larger proteins), while the precision in the direct dimension is decreasing with increasing $R_{2,HN}$.

Example 8 – Analytical Calculations Using the Gaussian Law of Error Propagation

[0128]   Three different classes of combinations are identified.

(I) 2 combinations provide high precision [$\sigma(X) = \frac{1}{2} \cdot \sigma(\text{basic})$; X = $^1H^\alpha$, $^{13}C^\alpha$, $^{13}C'$, ^{15}N] for all four chemical shifts:

$B1$ [$\Omega_0 + \Omega_1 + \Omega_2 + \Omega_3$]; $B4$ [$\Omega_0 - \Omega_1 - \Omega_2 + \Omega_3$]; $B6$ [$\Omega_0 - \Omega_1 + \Omega_2 - \Omega_3$]; and $B7$ [$\Omega_0 + \Omega_1 - \Omega_2 - \Omega_3$], or

$B2$ [$\Omega_0 - \Omega_1 + \Omega_2 + \Omega_3$]; $B3$ [$\Omega_0 + \Omega_1 - \Omega_2 + \Omega_3$]; $B5$ [$\Omega_0 + \Omega_1 + \Omega_2 - \Omega_3$]; and $B8$ [$\Omega_0 - \Omega_1 - \Omega_2 - \Omega_3$].

(II) 26 combinations provide intermediate precision [$\sigma(X) = \frac{1}{\sqrt{2}} \cdot \sigma(\text{basic})$; X = $^1H^\alpha$, $^{13}C^\alpha$, $^{13}C'$, ^{15}N] for all four chemical shifts.

(III) 37 combinations provide intermediate precision [$\sigma = \frac{1}{\sqrt{2}} \cdot \sigma(\text{basic})$] for three of the shifts and low precision [$\sigma = \sigma(\text{basic})$] for one of the four shifts.

The standard deviation depends on the number of equations that need to be linearly combined to calculate the shifts. This can be discussed for three examples, one representing each of the cases.

(I) $B2$ [$\Omega_0 - \Omega_1 + \Omega_2 + \Omega_3$]; $B3$ [$\Omega_0 + \Omega_1 - \Omega_2 + \Omega_3$]; $B5$ [$\Omega_0 + \Omega_1 + \Omega_2 - \Omega_3$]; and

$B8$ [Ω_0 - Ω_1 - Ω_2 - Ω_3] are selected. Then, the individual chemical shifts are obtained from:

$4 \Omega_0(^{15}N) = B2 + B3 + B5 + B8$

$4 \Omega_1(^{13}C') = -B2 + B3 + B5 - B8$

5    $4 \Omega_2(^{13}C^\alpha) = B2 - B3 + B5 - B8$

$4 \Omega_3(^1H^\alpha) = B2 + B3 - B5 - B8$

with "BX" representing the shifts extracted from the spectrum BX (X = 2,3,5,8). Each shift from BX is associated with a standard deviation of σ(basic). Hence, the Gaussian law of error propagation (Eadie et al., Statistical Methods in Experimental

10   Physics, North-Holland, New York (1982), which is hereby incorporated by reference in its entirety) yields:

$\sigma[4 \Omega_0(^{15}N)] = 4 \sigma[\Omega_0(^{15}N)] = \sqrt{4}\ \sigma(\text{basic}) = 2 \cdot \sigma\,(\text{basic})$

$\sigma[4 \Omega_0(^{13}C')] = 4 \sigma[\Omega_0(^{13}C')] = \sqrt{4}\ \sigma(\text{basic}) = 2 \cdot \sigma\,(\text{basic})$

$\sigma[4 \Omega_0(^{13}C^\alpha)] = 4 \sigma[\Omega_0(^{13}C^\alpha)] = \sqrt{4}\ \sigma(\text{basic}) = 2 \cdot \sigma\,(\text{basic})$

15    $\sigma[4 \Omega_0(^1H^\alpha)] = 4 \sigma[\Omega_0(^1H^\alpha)] = \sqrt{4}\ \sigma(\text{basic}) = 2 \cdot \sigma\,(\text{basic}),$

or equivalently,

$\sigma[\Omega_0(X)] = \dfrac{1}{\sqrt{4}}\ \sigma(\text{basic}) = \dfrac{1}{2} \cdot \sigma\,(\text{basic})$ for X = $^1H^\alpha$, $^{13}C^\alpha$, $^{13}C'$, ^{15}N.

Thus, the resulting precision is equivalent to the one obtained from four statistically independent measurements.

20

(II) $B1$ [Ω_0 + Ω_1 + Ω_2 + Ω_3]; $B5$ [Ω_0 + Ω_1 + Ω_2 - Ω_3]; $B7$ [Ω_0 + Ω_1 - Ω_2 - Ω_3]; and $B8$ [Ω_0 - Ω_1 - Ω_2 - Ω_3] are selected. Then, the individual chemical shifts are obtained from:

$2 [\Omega_0(^{15}N) = B1 + B8$

25    $2 \Omega_1(^{13}C') = B7 - B8$

$2 \Omega_2(^{13}C^\alpha) = B5 - B7$

$2 \Omega_3(^1H^\alpha) = B1 - B5$

with "BX" representing the shifts extracted from the spectrum BX (X = 1,5,7,8). Each shift from BX is associated with a standard deviation of σ(basic). Hence, the

30   Gaussian law of error propagation yields:

$\sigma[2 \Omega_0(^{15}N)] = 2 \sigma[\Omega_0(^{15}N)] = \sqrt{2}\ \sigma(\text{basic})$

$$\sigma[2\,\Omega_0(^{13}C')] = 2\,\sigma[\Omega_0(^{13}C')] = \sqrt{2}\ \sigma(\text{basic})$$

$$\sigma[2\,\Omega_0(^{13}C^\alpha)] = 2\,\sigma[\Omega_0(^{13}C^\alpha)] = \sqrt{2}\ \sigma(\text{basic})$$

$$\sigma[2\,\Omega_0(^{1}H^\alpha)] = 2\,\sigma[\Omega_0(^{1}H^\alpha)] = \sqrt{2}\ \sigma(\text{basic}),$$

or equivalently,

5　$\sigma[\Omega_0(X)] = \dfrac{1}{\sqrt{2}}\ \sigma(\text{basic})$ for $X = {}^{1}H^\alpha,\ {}^{13}C^\alpha,\ {}^{13}C',\ {}^{15}N.$

Thus, the resulting precision is equivalent to the one obtained from two statistically independent measurements.

(III)　$B1\ [\Omega_0 + \Omega_1 + \Omega_2 + \Omega_3]$;　$B4\ [\Omega_0 - \Omega_1 - \Omega_2 + \Omega_3]$;　$B5\ [\Omega_0 + \Omega_1 + \Omega_2 - \Omega_3]$;　and

10　$B6\ [\Omega_0 - \Omega_1 + \Omega_2 - \Omega_3]$ are selected. Then, the individual chemical shifts are obtained from:

$$2\ [\Omega_0(^{15}N) = B4 + B5$$

$$2\,\Omega_1(^{13}C') = B5 - B6$$

$$2\,\Omega_2(^{13}C^\alpha) = B1 - B4 - B5 + B6$$

15　$2\,\Omega_3(^{1}H^\alpha) = B1 - B5$

with "BX" representing the shifts extracted from the spectrum BX ($X = 1,4,5,6$). Each shift from BX is associated with a standard deviation of $\sigma(\text{basic})$. Hence, the Gaussian law of error propagation yields:

$$\sigma[2\,\Omega_0(^{15}N)] = 2\,\sigma[\Omega_0(^{15}N)] = \sqrt{2}\ \sigma(\text{basic})$$

20　$\sigma[2\,\Omega_0(^{13}C')] = 2\,\sigma[\Omega_0(^{13}C')] = \sqrt{2}\ \sigma(\text{basic})$

$$\sigma[2\,\Omega_0(^{13}C^\alpha)] = 2\,\sigma[\Omega_0(^{13}C^\alpha)] = \sqrt{4}\ \sigma(\text{basic}) = 2\cdot\sigma\ (\text{basic})$$

$$\sigma[2\,\Omega_0(^{1}H^\alpha)] = 2\,\sigma[\Omega_0(^{1}H^\alpha)] = \sqrt{2}\ \sigma(\text{basic}),$$

or equivalently,

$$\sigma[\Omega_0(X)] = \dfrac{1}{\sqrt{2}}\ \sigma(\text{basic}) \text{ for } X = {}^{1}H^\alpha, {}^{13}C', {}^{15}N \text{ and}$$

25　$\sigma[\Omega_0(^{13}C^\alpha)] = \sigma(\text{basic})$

Thus, the resulting precision is equivalent to the one obtained from two statistically independent measurements for three of the chemical shifts, while it is equivalent to a single measurement for one of the shifts.

[0129]　　In case all 15 spectra constituting the constant time (5,2)D GFT NMR

30　experiment are selected, similar considerations show that the resulting standard

deviations can be calculated (Eadie et al., <u>Statistical Methods in Experimental Physics</u>, North-Holland, New York (1982), which is hereby incorporated by reference in its entirety) according to the following equations.

(a) Survey of constant time spectra, standard deviations and chemical shift measurements

Data	Number of Spectra	Standard Deviation	Chemical Shift Measurements
Basic	8	σ(basic)	$\Omega_0(^{15}N) \pm \Omega_1(^{13}C') \pm \Omega_2(^{13}C^\alpha) \pm \Omega_3(^1H^\alpha)$
1st	4	σ(1st)	$\Omega_0(^{15}N) \pm \Omega_1(^{13}C') \pm \Omega_2(^{13}C^\alpha)$
2nd	2	σ(2nd)	$\Omega_0(^{15}N) \pm \Omega_1(^{13}C')$
3rd	1	σ(3rd)	$\Omega_0(^{15}N)$

(b) Calculation of error propagation

Chemical Shifts	Standard Deviations
$\Omega_0(^{15}N)$	$\sigma(^{15}N) = \dfrac{\sqrt{8 \cdot \sigma^2(basic) + 4 \cdot \sigma^2(1st) + 2 \cdot \sigma^2(2nd) + \sigma^2(3rd)}}{15}$
$\Omega_1(^{13}C')$	$\sigma(^{13}C') = \dfrac{\sqrt{8 \cdot \sigma^2(basic) + 4 \cdot \sigma^2(1st) + 2 \cdot \sigma^2(2nd)}}{14}$
$\Omega_2(^{13}C^\alpha)$	$\sigma(^{13}C^\alpha) = \dfrac{\sqrt{8 \cdot \sigma^2(basic) + 4 \cdot \sigma^2(1st)}}{12}$
$\Omega_3(^1H^\alpha)$	$\sigma(^1H^\alpha) = \dfrac{\sqrt{8 \cdot \sigma^2(basic)}}{8}$

The validity of these equations is neatly confirmed by the Monte Carlo simulation performed with input from all 15 spectra:

σ	6σ (simulated)	6σ (calculated)
$\sigma(^{15}N)$	14.50	14.46
$\sigma(^{13}C')$	15.35	15.37
$\sigma(^{13}C^\alpha)$	17.41	17.36
$\sigma(^1H^\alpha)$	21.24	21.26

Example 9 – Implementation of the (5,2)D HACA,CONHN, (5,3)D HACA,CONHN / (5,3)D HACACONHN, and (4,3)D CBCACONHN / (4,3)D CBCA,CONHN GFT NMR Experiments

[0130] The following GFT NMR experiments were implemented (Figure 4): (i) with K = 3, (5,2)D HACA,CONHN complementing (5,2)D HACACONHN for sequential assignment, (ii) with K = 2, (5,3)D HACA,CONHN and (5,3)D HACACONHN, where, in contrast to the (5,2)D experiments in (i), the ^{15}N chemical shifts evolve separately, and (iii) with K = 1, (4,3)D CBCACONHN and (4,3)D CBCA,CONHN. The underlined letters indicate which chemical shifts that are jointly sampled. After G-matrix transformation one obtains $2^{3+1} - 1 = 15$ 2D planes for the (5,2)D experiments (K = 3), seven 3D spectra for the (5,3)D experiments (K = 2) and three 3D spectra for the (4,3)D experiments (K = 1). The magnetization transfer pathways are depicted in Figures 4A-C. Spectra were acquired for the 8.6 kDa protein ubiquitin and for the 14 kDa protein TT212 from the protein structure production pipeline of the Northeast Structural Genomics Consortium (http://www.nesg.org, which is hereby incorporated by reference in its entirety).

[0131] (5,2)D HACA,CONHN / (5,2)D HACACONHN and (5,3)D HACA,CONHN / (5,3)D HACACONHN correlate the backbone amide ^{15}N and ^{1}HN chemical shifts of residue i with the ^{13}C', ^{13}C$^{\alpha}$ and ^{1}H$^{\alpha}$ chemical shifts of residue i-1 and i, respectively, via one-bond scalar couplings (Figures 4A-B). In addition, the often smaller two-bond scalar couplings between the ^{15}N$_i$ and ^{13}C$^{\alpha}_{i-1}$ may yield sequential connectivities in the HACA,CONHN experiments. The comma separating "CA" from "CO" indicates that the intraresidue ^{13}C' chemical shift is obtained by creating two-spin coherence involving ^{13}C$^{\alpha}$ and ^{13}C' during the intraresidue polarization transfer from ^{13}C$^{\alpha}$ to ^{15}N (Löhr et al., J. Biomol. NMR 6:189–197 (1995), which is hereby incorporated by reference in its reference). The (5,2)D HACA,CONHN experiment was recorded with the radio-frequency (rf) pulse scheme of Fig. 7A. Löhr et al., J. Biomol. NMR 6:189–197 (1995), which is hereby incorporated by reference in its entirety, can be referred to for a product operator description of the experiment. Since rf pulses on ^{13}C' are employed as laminar shifted pulses (Cavanagh et al., Protein NMR Spectroscopy, Wiley, New York (1996), which

is hereby incorporated by reference in its entirety), the spectral width of the indirect dimension was set to one half of the difference of the $^{13}C^{\alpha}$ and $^{13}C'$ carrier frequencies (8,897 Hz at 600 MHz) in order to fold the $^{13}C'$ onto the $^{13}C^{\alpha}$ carrier frequency. In the current implementation of (5,2)D HACA,CONHN, $\Omega(^{15}N)$ was detected in quadrature in the GFT dimension ω_1. With the GFT NMR super phase-cycle given in the legend of Fig. 7A, this yields (i) eight basic 2D spectra with peaks at $\Omega_0(^{15}N)\pm\Omega_1(^{13}C')\pm\Omega_2(^{13}C^{\alpha})\pm\Omega_3(^1H^{\alpha})$ along ω_1, (ii) four 2D first order central peak spectra with peaks at $\Omega_0(^{15}N)\pm\Omega_1(^{13}C')\pm\Omega_2(^{13}C^{\alpha})$, (iii) two 2D second order central peak spectra with peaks at $\Omega_0(^{15}N)\pm\Omega_1(^{13}C')$ and (iv) one 2D third order central peak spectrum with peaks at $\Omega_0(^{15}N)$. The choice for the order of central peak detection is primarily guided by sensitivity considerations. First order central peaks were derived from $^{13}C^{\alpha}$ magnetization, which allows one to detect these central peaks while the basic spectra are acquired. Hence, when the basic spectra are acquired with at least two scans per increment, the first order central peaks are obtained from ^{13}C steady state magnetization without investment of additional measurement time. In case single scan acquisition is chosen for the basic spectra, first order central peak detection would be best implemented by simply omitting the $^1H^{\alpha}$ shift evolution. Second order central peak were derived from $^1H^{\alpha}$ magnetization using the scheme of Fig. 7A, i.e., by omitting both the $^1H^{\alpha}$ and $^{13}C^{\alpha}$ chemical shift evolution periods. This approach is more sensitive than using 2D HNN(CA)CO. Finally, sensitive 2D [$^{15}N,^1H$]-HSQC provided the third order central peaks. To match (5,2)D HACA,CONHN, (5,2)D HACACONHN (Fig. 4A) was acquired with the same order for central peak detection as in (5,2)D HACA,CONHN, except that the spectral width of the indirect GFT dimension was set to 8,897 Hz.

[0132]   (5,3)D HACACONHN and HACA,CONHN were recorded using the pulse scheme and a correspondingly reduced GFT NMR super phase cycle of the (5,2)D congeners (Figure 7A); $\Omega(^{13}C')$ was detected in quadrature in the GFT dimension and $\Omega(^{15}N)$ was sampled in a separate chemical shift evolution along ω_2. This yields (i) four basic 3D spectra with peaks at $\Omega_0(^{13}C')\pm\Omega_1(^{13}C^{\alpha})\pm\Omega_2(^1H^{\alpha})$, (ii) two first order central peak spectra with peaks at $\Omega_0(^{13}C')\pm\Omega_1(^{13}C^{\alpha})$ and (ii) one second order central peak spectrum with peaks at $\Omega_0(^{13}C')$.

[0133] (4,3)D <u>CBCACO</u>NHN and (4,3)D <u>CBCA,CO</u>NHN correlate the backbone amide ^{15}N and ^{1}HN chemical shifts of residue i with the ^{13}C', ^{13}C$^{\alpha}$ and ^{13}C$^{\beta}$ chemical shifts of residue i-1 and i, respectively, via one-bond scalar couplings (Figure 4C), and the often smaller two-bond scalar couplings between the ^{15}N$_i$ and ^{13}C$^{\alpha}_{i-1}$ may yield additional sequential connectivities in (4,3)D <u>CBCA,CO</u>NHN. $\Omega(^{13}$C') was detected in quadrature in the GFT dimension thus yielding (i) two basic 3D spectra with peaks at $\Omega_0(^{13}$C')$\pm\Omega_1(^{13}$C$^{\alpha})$ and $\Omega_0(^{13}$C')$\pm\Omega_1(^{13}$C$^{\beta})$ and (ii) one central peak spectrum with peaks at $\Omega_0(^{13}$C'). (4,3)D <u>CBCACO</u>NHN was recorded by modifying the <u>H</u>$^{\alpha/\beta}$<u>C</u>$^{\alpha/\beta}$(CO)NHN pulse scheme (derived from CBCA(CO)NHN; Grzesiek et al., <u>J. Am. Chem. Soc.</u> 114:6291–6293 (1992), which is hereby incorporated by reference in its entirety) described in Szyperski et al., <u>Proc. Natl. Acad. Sci. USA</u> 99:8009–8014 (2002), which is hereby incorporated by reference in its entirety: the ^{1}H$^{\alpha/\beta}$ chemical shift evolution was eliminated and a ^{13}C' chemical shift evolution was introduced in a constant-time manner (see Figure 8 for the rf pulse scheme). (4,3)D <u>CBCA,CO</u>NHN was recorded with the new pulse scheme shown in Figure 7B, that is, ^{13}C'–^{13}C$^{\alpha}$ two-spin coherence is created for simultaneous ^{13}C' and ^{13}C$^{\alpha}$ frequency labeling during the polarization transfer from ^{13}C$^{\alpha}$ to ^{15}N.

Example 10 – Analyses of the (5,2)D <u>HACA,CO</u>NHN, (5,3)D <u>HACA,CO</u>NHN / (5,3)D <u>HACACO</u>NHN, and (4,3)D <u>CBCACO</u>NHN / (4,3)D <u>CBCA,CO</u>NHN GFT NMR Experiments

[0134] On a VARIAN Inova 600 MHz spectrometer at 25°C, (i) (5,2)D <u>HACACO</u>NHN (2.5 hrs. measurement time), (5,2)D <u>HACA,CO</u>NHN (8.1 hrs.), (ii) (5,3)D <u>HACACO</u>NHN (10.4 hrs.) and (5,3)D <u>HACA,CO</u>NHN (10.4 hrs.), and (iii) (4,3)D <u>CBCACO</u>NHN (5.6 hrs.) and (4,3)D <u>CBCA,CO</u>NHN (5.6 hrs.) were acquired for a 2 mM solution (pH = 5.8, 50 mM K-PO$_4$, 90% H2O/10% D$_2$O) of the 8.6 kDa protein ubiquitin. (5,3)D <u>HACACO</u>NHN (20.8 hrs.) and (5,3)D <u>HACA,CO</u>NHN (41.8 hrs) were also acquired for a 1 mM solution (pH = 6.5, 450 mM NaCl, 10 mM DTT, 20 mM Zn^{2+}, 0.01% NaN$_3$, 95% H$_2$O/5% D$_2$O) of the 14 kDa protein structural genomics target protein TT212.

[0135]   The yield of peak detection, i.e. the ratio of observed peaks over the total number of expected peaks, was (virtually) complete throughout. Reductions in minimal measurement time, ε, achievable in GFT NMR are given by the ratio of the number of free induction decays (FIDs) of an $(N,N\text{-}K)$D GFT NMR experiment over and the number FIDs of the ND FT NMR experiment.

[0136]   For ubiquitin, the following was obtained: (i) (5,2)D HACACONHN (ε = 225; 100% yield; S/N for peaks in basic spectra: 6.9 - 14.8; in first order central peak spectra: 8.1 - 10.4), (5,2)D HACA,CONHN (ε = 225; intraresidue correlations: 100% yield; S/N for peaks in basic spectra: 4.0 - 6.8; in first order central peak spectra: 3.3 - 5.3), (ii) (5,3)D HACACONHN (ε = 25; 100% yield; S/N for peaks in basic spectra: 27.5 - 61.2; in first order central peak spectra: 26.2 - 41.3) and (5,3)D HACA,CONHN (ε = 25; intraresidue correlations: 100% yield; S/N for peaks in basic spectra: 14.7 - 23.6; 93% in first order central peak spectra: 13.1 - 22.6), (iii) (4,3)D CBCACONHN (ε = 6.4; $^{13}C^{\alpha}$ correlations in basic spectra: 100% yield; S/N: 31.1 - 72.3; $^{13}C^{\beta}$ correlations in basic spectra: 100% yield; S/N: 23.8 - 81.1) and (4,3)D CBCA,CONHN (ε = 6.4; intraresidue $^{13}C^{\alpha}$ correlations in basic spectra: 100% yield; S/N: 3.7 - 23.9; intraresidue $^{13}C^{\beta}$ correlations in basic spectra: 99% yield; S/N: 2.7 - 9.7).

[0137]   For TT212, the following was obtained: (5,3)D HACACONHN (ε = 25; 100% yield; S/N for peaks in basic spectra: 3.0 - 44.6; in first order central peak spectra: 2.5 - 34.4) and (5,3)D HACA,CONHN (ε = 25; intraresidue correlations: 96% yield; S/N for peaks in basic spectra: 1.5 - 14.0; 93% in first order central peak spectra: 1.5 - 14.7). (S/N ratios not reported for other central peak spectra are larger than those of the first order central peaks.)

[0138]   When using (5,2)D HACACONHN / HACA,CONHN (Fig. 21) or (5,3)D HACACONHN and HACA,CONHN (Fig. 22), the sequential assignment is based on the three chemical shifts $\Omega(^{13}C'), \Omega(^{13}C^{\alpha})$ and $\Omega(^{1}H^{\alpha})$. The use of (4,3)D CBCACONHN / (4,3)D CBCA,CONHN (Figure 23) corresponds to having two 4D experiments in which the number of correlations is increased by a $^{13}C^{\beta}\text{-}^{13}C^{\alpha}$ relay step. Hence, the (4,3)D experiments likewise provide assignments based on three chemical shifts, i.e. $\Omega(^{13}C'), \Omega(^{13}C^{\alpha})$ and $\Omega(^{13}C^{\beta})$. Note, however, that $\Omega(^{13}C^{\alpha})$

and $\Omega(^{13}C^{\beta})$ of a given residue are not directly correlated. Figures 21-23 show that the exhaustive sampling of linear combinations of chemical shifts yields an extended set of sequential connectivities when compared with conventional FT NMR. For example, in (5,3)D HACACONHN / HACA,CONHN seven peaks located at $\Omega_0(^{13}C') \pm \Omega_1(^{13}C^{\alpha}) \pm \Omega_2(^{1}H^{\alpha})$ (spectra B1 to B4 in Figure 22), $\Omega_0(^{13}C') \pm \Omega_1(^{13}C^{\alpha})$ (spectra B5 and B6 in Figure 22) and $\Omega_1(^{13}C')$ (spectrum B7 in Figure 22) serve as sequential matching constraints. Recording of 3D HA(CACO)NHN, 3D (HA)CA(CO)NHN and 3D (HACA)CONHN spectra in conjunction with their intraresidue counterparts would yield only 3 constraints, which are devoid of direct correlations between the shifts of $^{13}C'$, $^{13}C^{\alpha}$ and $^{1}H^{\alpha}$ (as provided by the (5,3)D GFT NMR experiment).

[0139]   Furthermore, the experimental error of chemical shift measurements in constant time GFT NMR experiments scales with $1/(\sqrt{n})$, where n is the number of linear combinations contributing to the determination of a shift (assuming, for simplicity, that the same maximal evolution times have been chosen). The increased accuracy of the measurement is documented by comparing the shifts of the same nuclei measured in intra- and interresidue GFT data. Tables 3 to 5 afford a detailed analysis of the shift measurements associated with sequential connectivities shown in Figures 21 to 23, respectively. These tables provide both the measured linear combinations of shifts and the single-quantum shifts obtained from a linear least-squares fit. The experimental errors for the measurement of the linear combinations of the chemical shifts were estimated as described above. The comparison of shifts for the same nucleus as obtained from two different GFT NMR spectra shows that the accuracy is indeed high: the shift differences in Tables 3 to 5 (underlined values; see also Table 6 with the shift analysis corresponding to Figure 24) are smaller than 0.081 ppm for all cases in (5,3)D and (5,2)D GFT NMR spectra, and smaller than 0.154 ppm in the (4,3)D spectra.

Table 3. Chemical Shifts Measured in (5,2)D <u>HACA,CONHN</u> / (5,2)D <u>HACACONHN</u> Recorded for Ubiquitin (see Figure 21). The Underlined Values in the Lower Right Represent the Differences of Single-Quantum Shifts Obtained from (5,2)D <u>HACA,CONHN</u> and (5,2)D <u>HACACONHN</u>.

(A) (5,2)D <u>HACA,CONHN</u>

Linear combinations of shifts (Glu 64) measured along ω_1 [ppm]

$\Omega_0(^{15}N) + \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	108.075 ± 0.167	B1
$\Omega_0(^{15}N) - \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	103.697 ± 0.167	B2
$\Omega_0(^{15}N) + \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	97.120 ± 0.167	B3
$\Omega_0(^{15}N) - \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	92.937 ± 0.167	B4
$\Omega_0(^{15}N) + \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	136.303 ± 0.167	B5
$\Omega_0(^{15}N) - \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	131.989 ± 0.167	B6
$\Omega_0(^{15}N) + \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	125.512 ± 0.167	B7
$\Omega_0(^{15}N) - \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	121.182 ± 0.167	B8
$\Omega_0(^{15}N) + \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha)$	122.043 ± 0.167	B9
$\Omega_0(^{15}N) - \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha)$	117.721 ± 0.167	B10
$\Omega_0(^{15}N) + \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha)$	111.432 ± 0.167	B11
$\Omega_0(^{15}N) - \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha)$	107.122 ± 0.167	B12
$\Omega_0(^{15}N) + \Omega_1(^{13}C')$	116.832 ± 0.106	B13
$\Omega_0(^{15}N) - \Omega_1(^{13}C')$	112.351 ± 0.106	B14
$\Omega_0(^{15}N)$	114.577 ± 0.078	B15

Single-quantum shifts [ppm]

$\Omega_0(^{15}N)$	114.593 ± 0.040
$\Omega_1(^{13}C')$	175.133 ± 0.017
$\Omega_2(^{13}C^\alpha)$	58.427 ± 0.019
$\Omega_3(^1H^\alpha)$	3.347 ± 0.006

Table 3 (continued)

(B) (5,2)D HACACONHN

Linear combinations of shifts [$\Omega_0(^{15}N)$ of Ser 65 and of Glu 64 otherwise] measured along ω_1 [ppm]

$\Omega_0(^{15}N) + \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	108.510 ± 0.167	B1
$\Omega_0(^{15}N) - \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	103.758 ± 0.167	B2
$\Omega_0(^{15}N) + \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	97.642 ± 0.167	B3
$\Omega_0(^{15}N) - \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) + \Omega_3(^1H^\alpha)$	92.982 ± 0.167	B4
$\Omega_0(^{15}N) + \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	136.891 ± 0.167	B5
$\Omega_0(^{15}N) - \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	131.088 ± 0.167	B6
$\Omega_0(^{15}N) + \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	125.054 ± 0.167	B7
$\Omega_0(^{15}N) - \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha) - \Omega_3(^1H^\alpha)$	121.270 ± 0.167	B8
$\Omega_0(^{15}N) + \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha)$	122.727 ± 0.167	B9
$\Omega_0(^{15}N) + \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha)$	111.894 ± 0.167	B10
$\Omega_0(^{15}N) - \Omega_1(^{13}C') + \Omega_2(^{13}C^\alpha)$	117.984 ± 0.167	B11
$\Omega_0(^{15}N) - \Omega_1(^{13}C') - \Omega_2(^{13}C^\alpha)$	107.206 ± 0.167	B12
$\Omega_0(^{15}N) + \Omega_1(^{13}C')$	116.198 ± 0.106	B13
$\Omega_0(^{15}N) - \Omega_1(^{13}C')$	112.608 ± 0.106	B14
$\Omega_0(^{15}N)$	114.886 ± 0.078	B15

Single-quantum shifts [ppm]

$\Omega_0(^{15}N)$ (Ser 65)	114.913 ± 0.040	
$\Omega_1(^{13}C')$	175.210 ± 0.017	−0.077
$\Omega_2(^{13}C^\alpha)$	58.440 ± 0.019	−0.013
$\Omega_3(^1H^\alpha)$	3.344 ± 0.006	+0.003

Table 4. Chemical Shifts Measured in (5,3)D HACA,CONHN / (5,3)D HACACONHN Recorded for TT212 (see Figure 22). The Underlined Values in the Lower Right Represent the Differences of Single-Quantum Shifts Obtained from (5,3)D HACA,CONHN and (5,3)D HACACONHN.

(A) (5,3)D HACA,CONHN

Linear combinations of shifts (Ile 25) measured along ω_1 [ppm]

$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	180.354 ± 0.067	$B1$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	167.916 ± 0.067	$B2$
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha))$	185.688 ± 0.067	$B3$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$	173.824 ± 0.067	$B4$
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha)$	183.169 ± 0.067	$B5$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha)$	170.699 ± 0.067	$B6$
$\Omega_0(^{13}C')$	177.140 ± 0.045	$B7$

Single-quantum shifts [ppm]

$\Omega_0(^{13}C')$	176.970 ± 0.024
$\Omega_1(^{13}C^\alpha)$	62.389 ± 0.027
$\Omega_2(^1H^\alpha)$	4.073 ± 0.009

(B) (5,3)D HACACONHN

Linear combinations of shifts (Ile 25) measured along ω_1 [ppm]

$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	180.223 ± 0.067	$B1$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	168.256 ± 0.067	$B2$
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha))$	185.907 ± 0.067	$B3$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$	173.603 ± 0.067	$B4$
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha)$	183.052 ± 0.067	$B5$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha)$	170.933 ± 0.067	$B6$
$\Omega_0(^{13}C')$	177.075 ± 0.045	$B7$

Single-quantum shifts [ppm]

$\Omega_0(^{13}C')$	177.007 ± 0.024	−0.037
$\Omega_1(^{13}C^\alpha)$	62.325 ± 0.027	+0.064
$\Omega_2(^1H^\alpha)$	4.087 ± 0.009	−0.014

Table 5. Chemical Shifts Measured in (4,3)D $\underline{CB,CACO}$ NHN / (4,3)D $\underline{CBCACO}$ NHN Recorded for Ubiquitin (see Figure 23). The Underlined Values in the Lower Right Represent the Differences of Single-Quantum Shifts Obtained from (4,3)D $\underline{CB,CACO}$ NHN and (4,3)D $\underline{CBCACO}$ NHN.

(A) (4,3)D $\underline{CBCA,CO}$ NHN

Linear combinations of shifts (Ser 65) measured along ω_1 [ppm]

$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha)$	191.359 ± 0.067	$B1$
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\beta)$	195.451 ± 0.067	$B1$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha)$	152.401 ± 0.067	$B2$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\beta)$	148.249 ± 0.067	$B2$
$\Omega_0(^{13}C')$	171.848 ± 0.045	$B3$

Single-quantum shifts [ppm]

$\Omega_0(^{13}C')$	171.862 ± 0.027
$\Omega_1(^{13}C^\alpha)$	60.789 ± 0.047
$\Omega_1(^{13}C^\beta)$	64.911 ± 0.047

(B) (4,3)D $\underline{CBCACO}$ NHN

Linear combinations of shifts (Ser 65) measured along ω_1 [ppm]

$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha)$	191.533 ± 0.067	$B1$
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\beta)$	195.530 ± 0.067	$B1$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha)$	152.267 ± 0.067	$B2$
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\beta)$	148.225 ± 0.067	$B2$
$\Omega_0(^{13}C')$	171.887 ± 0.045	$B3$

Single-quantum shifts [ppm]

$\Omega_0(^{13}C')$	171.888 ± 0.027	$\underline{-0.026}$
$\Omega_1(^{13}C^\alpha)$	60.943 ± 0.047	$\underline{-0.154}$
$\Omega_1(^{13}C^\beta)$	64.962 ± 0.047	$\underline{-0.051}$

Table 6. Chemical Shifts Measured in (5,3)D <u>HACA,CO</u>NHN / (5,3)D <u>HACACO</u>NHN Recorded for Ubiquitin (see Figure 24). The Underlined Numbers in the Lower Right Represent the Differences of Single-Quantum Shifts Obtained from (5,3)D <u>HACA,CO</u>NHN and (5,3)D<u>HACACO</u>NHN.

(A) (5,3)D <u>HACA,CO</u>NHN

Linear combinations of shifts (Ser 65) measured along ω_1 [ppm] (Figure 21)

$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	176.020 ± 0.067	B1
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	166.513 ± 0.067	B2
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$	177.233 ± 0.067	B3
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$	167.684 ± 0.067	B4
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha)$	176.656 ± 0.067	B5
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha)$	167.090 ± 0.067	B6
$\Omega_0(^{13}C')$	171.848 ± 0.045	B7

Single-quantum shifts [ppm]

$\Omega_0(^{13}C')$	171.863 ± 0.024
$\Omega_1(^{13}C^\alpha)$	61.030 ± 0.027
$\Omega_2(^1H^\alpha)$	4.630 ± 0.009

(B) (5,3)D <u>HACACO</u>NHN [ubiquitin]

Linear combinations of shifts (Ser 65) measured along ω_1 [ppm] (Figure 21)

$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	175.975 ± 0.067	B1
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha))$	177.140 ± 0.067	B2
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) - \Omega_2(^1H^\alpha)$	167.770 ± 0.067	B3
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha) + \Omega_2(^1H^\alpha)$	166.610 ± 0.067	B4
$\Omega_0(^{13}C') + \Omega_1(^{13}C^\alpha)$	176.598 ± 0.067	B5
$\Omega_0(^{13}C') - \Omega_1(^{13}C^\alpha)$	167.197 ± 0.067	B6
$\Omega_0(^{13}C')$	171.887 ± 0.045	B7

Single-quantum shifts [ppm]

$\Omega_0(^{13}C')$	171.882 ± 0.024	<u>-0.019</u>
$\Omega_1(^{13}C^\alpha)$	60.949 ± 0.027	<u>+0.081</u>
$\Omega_2(^1H^\alpha)$	4.634 ± 0.009	<u>-0.004</u>

[0140]   Automated resonance assignment (Szyperski et al., J. Biomol. NMR 11:387–405 (1998); Moseley et al., Meth. Enzymol. 339:91–108 (2002), which are hereby incorporated by reference in their entirety), for high-throughput structure determination in structural genomics (Montelione et al., Nature Struc. Biol. 7:982–984 (2000), which is hereby incorporated by reference in its entirety) may profit from employment of GFT NMR in either of the two ways described in the following. First, peak lists of GFT NMR spectra may be used directly to establish sequential connectivities. Then, the extended set of connectivities (see Figures 21-23) corresponding to the matching of 2^m-1 "linear combinations" of shifts is redundant and contains ND information. Notably, automated resonance assignment protocols are rather sensitive to the lack of even a smaller number of sequential connectivities. Hence, one can expect to establish more reliable strategies when compared to the use of conventional spectroscopy, also for smaller proteins with molecular weights around 10 kDa. Alternatively, the GFT NMR peak lists can be used to calculate ND peak lists containing precise single-quantum shifts. Subsequently, sequential connectivities are established based on matching of single-quantum shifts. Due to the increased accuracy of the GFT shift measurements, correspondingly reduced matching tolerances (defined as the chemical shift difference between two shift values below which these are considered to be identical) can be employed. For example, the program AUTOASSIGN (Monleon et al., J. Struc. Func. Genomics 2:93–101 (2002), which is hereby incorporated by reference in its entirety) is routinely executed with matching tolerances of 0.4 ppm for $^{13}C^{\alpha/\beta}$ shifts, 0.25 ppm for $^{13}C'$ shifts and 0.04 ppm for $^{1}H^{\alpha}$ shifts measured in indirect dimensions of FT triple resonance NMR spectra. Setting the matching tolerance for analysis of (5,2)D and (5,3)D GFT NMR derived ND peak list to about 2 times the maximal shift difference (Tables 3 to 5), one obtains as a first estimate: ~0.15 ppm for $^{13}C^{\alpha}$ shifts, ~0.15 ppm for $^{13}C'$ shifts and ~0.02 ppm for $^{1}H^{\alpha}$ shifts. Future statistical analysis of several GFT NMR spectra and the use of AUTOASSIGN for GFT NMR data have to reveal the magnitude of the reduction of matching tolerances more accurately, but the estimates presented herein clearly show that a significant reduction can be anticipated. Notably, the accuracy of shift measurements using constant time GFT NMR experiments is independent of

transverse relaxation rates (which solely determine the peak intensity) and thus independent of the molecular weight.

[0141]   In principle, with respect to the detection of sequential peaks in the experiments providing the intraresidue connectivities, one may "filter out" the sequential connectivities (e.g., Brutscher, J. Magn. Reson. 156:155–159 (2002), which is hereby incorporated by reference in its entirety). However, for some applications, it is preferable not to eliminate sequential peaks, since (i) such filtering compromises on the sensitivity, (ii) the sequential peaks can be readily identified in the sequential congener, and (iii) the sequential peaks in the intraresidue experiment can be used to accurately adjust the calibration of the two GFT NMR spectra used in conjunction. For automated assignment protocols, the procedure of point (iii) is of outstanding value to reduce matching tolerances and is thus routinely employed. At highest magnetic fields (900 MHz ^{1}H resonance frequency), it might be advantageous to design GFT experiments providing the sequential connectivities in a manner suggested by Meissner et al., J. Magn. Reson. 150:100–104 (2001), which is hereby incorporated by reference in its entirety.

[0142]   In view of the introduction of cryogenic probes, which reduce NMR measurement times by a factor of 10 or more (Monleon et al., J. Struc. Func. Genomics 2:93–101 (2002), which is hereby incorporated by reference in its entirety), GFT NMR experiments providing 4D and 5D NMR spectral information are highly attractive also for larger systems. For example, (5,2)D HACACONHN / HACA,CONHN and (4,3)D CBCACONHN / CBCA,CONHN were acquired in only 10.6 and 11.2 hours, respectively, for an 8.6 kDa protein and it can thus be expected that similarly short measurement times are feasible for medium-sized protein up to about 20 kDa when using cryogenic probes. In fact, the (5,3)D data sets of 14 kDa TT212 (Figure 22) were acquired in about 60 hours, so that the same data could have been recorded within a few hours with a cryogenic probe. Apart from sensitivity, spectral resolution is critical for employment of multidimensional NMR. At high magnetic fields, (4,3)D and (5,3)D ^{15}N-resolved GFT NMR experiments are well suited to approach large proteins, at least to the extent such conventional ^{15}N-resolved 3D spectra are currently used. For the (5,2)D experiments, one needs to consider that peak dispersion increases linearly from third order (2D [^{15}N,^{1}H]-HSQC), to second

order to first order and to basic spectra (see Figure 17). Moreover, future research needs to show how effectively computer supported "bottom-up" identification of chemical shift multiplets restores the 5D dispersion. Certainly, the dispersion of the 2D [^{15}N,^{1}H]-HSQC provides a good initial indication with respect to the degree of overlap that needs to be resolved during the "bottom-up" assignment. At 900 MHz ^{1}H resonance frequency, 20-25 kDa proteins often exhibit rather well resolved 2D [^{15}N,^{1}H]-TROSY (Pervushin et al., Proc. Natl. Acad. Sci. USA 99:8009- 8014 (1997), which is hereby incorporated by reference in its entirety) spectra, and thus it is expected that proteins up to a least 20 kDa might well be approached using (5,2)D GFT-TROSY NMR at such highest field strengths.

[0143]   Finally, future research will show to which extent the acquisition speed of GFT NMR can be further increase (Frydman et al., Proc. Natl. Acad. Sci. USA 99:15858–15862 (2002), which is hereby incorporated by reference in its entirety), or Hadamard-type sampling schemes (Kupce et al., J. Magn. Reson. Ser. A 105 310–315 (1993); Kupce et al., J. Biomol. NMR 25:349–354 (2003), which are hereby incorporated by reference in their entirety). Moreover, it is conceivable that the "sampling demand" of GFT NMR can be further reduced by (i) non-linear sampling (Schmieder et al., J. Biomol. NMR 4:483–490 (1994), which is hereby incorporated by reference in its entirety), (ii) the employment of the filter diagonalization approach for data processing (Wall et al., J. Chem. Phys. 112:8011–8022 (1995); Hu et al., J. Magn. Reson. 134:76–87 (1998), which are hereby incorporated by reference in their entirety), or (iii) the use of "three-way decomposition" (Gutmanas et al., J. Biomol. NMR 24:191–201 (2002), which is hereby incorporated by reference in its entirety).

Example 11 – Implementation and Analyses of the (4,3)D HNNCACBCA, (4,3)D HNN(CO)CACBCA / (4,3)D CBCACA(CO)NHN, (5,3)D HBHACBCACA(CO)NHN, (5,3)D HCC,CH-COSY, (5,3)D HBCBCGCDHD, and (4,2)D HCCH-COSY GFT NMR Experiments

[0144]   The following GFT NMR experiments were conducted for the assignment of polypeptide backbone and sidechain resonances: (i) (4,3)D HNNCACBCA GFT NMR experiment (Figure 5A), (ii) (4,3)D CBCACA(CO)NHN/(4,3)D HNN(CO)CACBCA GFT NMR experiments (Figure

5B), (iii) (5,3)D HBHACBCACA(CO)NHN GFT NMR experiment (Figure 5C), (iv) (5,3)D HCC,CH-COSY GFT NMR experiment (Figure 5D), (v) (5,3)D HBCBCGCDHD GFT NMR experiment (Figure 5E), (vi) (4,2)D HCCH-COSY GFT NMR experiment (Figure 5F), and (vii) (5,2)D HCCCH-COSY GFT NMR

5   experiment (Figure 5G).

[0145]   In the (4,3)D HNNCACBCA GFT NMR experiment, after independent frequency labeling of $^{13}C^\alpha$ and $^{13}C^\beta$ spins of both amino acid residues i and i-1 (hereinafter referred to as i/i-1), magnetization is transferred to the respective $^{13}C^\alpha_{i/i-1}$ spin, which is then frequency labeled and detected in quadrature in each of the 3D

10 spectra constituting the (4,3)D experiment. Thus, for a given $^{15}N_i$ and $^1H^N_i$ chemical shift, the 2 basic spectra comprise peaks at $\Omega_0(^{13}C^\alpha_i)\pm\Omega_1(^{13}C^{\alpha/\beta}_i)$ and $\Omega_0(^{13}C^\alpha_{i-1})\pm\Omega_1(^{13}C^{\alpha/\beta}_{i-1})$. The first order central peak spectrum for (4,3)D HNNCACBCA was acquired using a 3D HNNCA pulse sequence comprising peaks at $\Omega_0(^{13}C^\alpha_i)$ and $\Omega_0(^{13}C^\alpha_{i-1})$.

15 [0146]   In the (4,3)D HNN(CO)CACBCA / (4,3)D CBCACA(CO)NHN GFT NMR experiments, the same principle as described in the (4,3)D HNNCACBCA GFT NMR experiment was used, except that the 2 basic spectra comprise, for a given $^{15}N_i$ and $^1H^N_i$ chemical shift, peaks from only amino acid residue i-1 at $\Omega_0(^{13}C^\alpha_{i-1})\pm\Omega_1(^{13}C^{\alpha/\beta}_{i-1})$ chemical shifts. (4,3)D HNN(CO)CACBCA GFT NMR experiment is

20 an "out-and-back" type of experiment, while (4,3)D CBCACA(CO)NHN GFT NMR experiment is an "out-and-stay" type. The first order central peak spectrum for (4,3)D HNN(CO)CACBCA / (4,3)D CBCACA(CO)NHN GFT NMR experiments comprising peaks at $\Omega_0(^{13}C^\alpha_{i-1})$ was acquired using a 3D HNN(CO)CA pulse sequence.

25 [0147]   Using the above-described (4,3)D HNNCACBCA GFT NMR experiment and the (4,3)D HNN(CO)CACBCA / (4,3)D CBCACA(CO)NHN GFT NMR experiments in a combined fashion, one can sequentially assign residue pairs (i/i-1) in a polypeptide chain, as illustrated in Figure 25 for Glu 73 of the 17 kDa protein ER75. Figure 26 illustrates the sequential walk for residues Val 27 to Ile 30

30 of the 7 kDa protein GR2 using the (4,3)D HNNCACBCA GFT NMR experiment and

the (4,3)D HNN(CO)<u>CACBCA</u> / (4,3)D <u>CBCACA</u>(CO)NHN GFT NMR experiments.

[0148] Having obtained the chemical shifts of $^{13}C^{\alpha/\beta}$ spins for a given amino acid residue, the (5,3)D <u>HBHACBCACA</u>(CO)NHN GFT NMR experiment can be used to obtain $^{1}H^{\alpha/\beta}$ chemical shifts. In this experiment, frequency labeling of the $^{1}H^{\alpha/\beta}$ spin was carried out simultaneously with that of $^{13}C^{\alpha/\beta}$ spins. Thus, for a given $^{15}N_i$ and $^{1}H^{N}_i$ chemical shift, the 4 basic spectra comprise peaks at $\Omega_0(^{13}C^{\alpha}_{i-1})\pm\Omega_1(^{13}C^{\alpha}_{i-1})\pm\Omega_2(^{1}H^{\alpha}_{i-1})$ and $\Omega_0(^{13}C^{\alpha}_{i-1})\pm\Omega_1(^{13}C^{\beta}_{i-1})\pm\Omega_2(^{1}H^{\beta}_{i-1})$. The $^{13}C^{\alpha/\beta}$ steady-state magnetization was used to obtain the two first order central peak spectra comprising peaks at $\Omega_0(^{13}C^{\alpha}_{i-1})\pm\Omega_1(^{13}C^{\alpha/\beta}_{i-1})$. The second order central peak spectrum was acquired using a 3D HNN(CO)CA pulse sequence comprising peaks at $\Omega_0(^{13}C^{\alpha}_{i-1})$. Figure 27 illustrates peak patterns observed in the (5,3)D <u>HBHACBCACA</u>(CO)NHN GFT NMR spectra, as well as the identity in the peak patterns observed in the basic spectra of the (4,3)D <u>CBCACA</u>(CO)NHN GFT NMR experiment and the first order central peak spectra of the (5,3)D <u>HBHACBCACA</u>(CO)NHN GFT NMR experiment.

[0149] The information of $^{1}H^{\alpha/\beta}$ and $^{13}C^{\alpha/\beta}$ chemical shifts can be used to assign the more peripheral spins of the aliphatic sidechain of a given amino acid residue by employing the (5,3)D <u>HCC</u>,CH-COSY GFT NMR experiment. For a given $^{13}C_i$ and $^{1}H_i$ chemical shift, the 4 basic spectra comprise peaks at $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)\pm\Omega_2(^{1}H_i)$ and $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C^{coupled}_i)\pm\Omega_2(^{1}H^{coupled}_i)$. The peak pattern observed in the (5,3)D <u>HCC</u>,CH-COSY GFT NMR experiment is illustrated in Figure 28. ^{13}C steady-state magnetization was used to obtain the two first order central peak spectra comprising peaks at $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)$ and $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C^{coupled}_i)$. The second order central peak spectrum was acquired using a 3D (H)C,CH-COSY pulse sequence and comprises peaks at $\Omega_0(^{13}C_i)$. The same pulse scheme as used for the (5,3)D <u>HCC</u>,CH-COSY GFT NMR experiment can also be used for assigning aromatic sidechain resonances in proteins by tuning the constant time delay to a value suited for transferring magnetization between aromatic ^{13}C-spins.

[0150] Resonance assignments of aromatic sidechain spins can be achieved by using a (5,3)D H̲B̲C̲B̲C̲G̲C̲D̲HD GFT NMR experiment and (4,2)D H̲C̲C̲H-COSY GFT NMR experiment. In the (5,3)D H̲B̲C̲B̲C̲G̲C̲D̲HD GFT NMR experiment, for a given $^{13}C^{\delta}$ and $^{1}H^{\delta}$ chemical shift, the 4 basic spectra comprise peaks at $\Omega_0(^{13}C^{\delta})\pm\Omega_1(^{13}C^{\beta})\pm\Omega_2(^{1}H^{\beta})$. In the (4,2)D H̲C̲C̲H-COSY GFT NMR experiment, for a given $^{1}H_i$ chemical shift, the 4 basic spectra comprise peaks at $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)\pm\Omega_2(^{1}H_i)$ and $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C^{coupled}_i)\pm\Omega_2(^{1}H_i^{coupled})$. The peak patterns observed in these spectra are illustrated in Figure 29 and 30, respectively, for the 8.6 kDa protein ubiquitin. The ^{13}C steady-state magnetization was used to obtain the two first order central peak spectra comprising peaks for the (5,3)D H̲B̲C̲B̲C̲G̲C̲D̲HD GFT NMR experiment at $\Omega_0(^{13}C^{\delta})\pm\Omega_1(^{13}C^{\beta})$ and for the (4,2)D H̲C̲C̲H-COSY GFT NMR experiment at $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)$ and $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i^{coupled})$. In the (5,3)D H̲B̲C̲B̲C̲G̲C̲D̲HD experiment, the chemical shift of the $^{13}C^{\delta}$ spin of aromatic amino acid residues was detected in quadrature along the ω_1 dimension in all the 3D spectra constituting the (5,3)D experiment. The second order central peak spectra for the (4,2)D H̲C̲C̲H-COSY and (5,3)D H̲B̲C̲B̲C̲G̲C̲D̲HD were acquired using the pulse sequence for a 2D $[^{13}C-^{1}H]$ HSQC (comprising peaks at $\Omega_0(^{13}C_i)$) and 3D (HBCB)CGCDHD-COSY (comprising peaks at $\Omega_0(^{13}C^{\delta})$), respectively. The same pulse scheme as used for the (4,2)D H̲C̲C̲H-COSY experiment can also be used for assigning aliphatic side-chain resonances in proteins by tuning the constant time delay to a value suited for transferring magnetization between aliphatic ^{13}C-spins.

[0151] The assignment of the side-chain chemical shifts can be further supported with the (5,2)D H̲C̲C̲C̲H-COSY experiment. In this experiment, for a $^{13}C_i$ and a $^{1}H_i$ chemical shift, the 8 basic spectra comprise peaks at: $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)\pm\Omega_2(^{13}C_i)\pm\Omega_3(^{1}H_i)$, $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)\pm\Omega_2(^{13}C_i^{coupled})\pm\Omega_3(^{1}H_i^{coupled})$, and $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i^{coupled})\pm\Omega_2(^{13}C_i^{coupled-2})\pm\Omega_3(^{1}H_i^{coupled-2})$. ^{13}C steady-state magnetization can be used to obtain the 4 first order central peak spectra comprising peaks at: $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)\pm\Omega_2(^{13}C_i)$, $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)\pm\Omega_2(^{13}C_i^{coupled})$, and $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i^{coupled})\pm\Omega_2(^{13}C_i^{coupled-2})$. The first order central peak spectra of (4,2)D H̲C̲C̲H-COSY represent the second order central peak spectra for the (5,2)D H̲C̲C̲C̲H-

COSY experiment, comprising peaks at: $\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i)$ and
$\Omega_0(^{13}C_i)\pm\Omega_1(^{13}C_i^{coupled})$. The third order central peak spectrum is acquired using a 2D
$[^{13}C\text{-}^1H]$ HSQC pulse sequence comprising peaks at $\Omega_0(^{13}C_i)$.

[0152]   The radiofrequency pulse schemes for the experiments described in
this example are shown in Figures 9-15. Tables 7-9 list the experiments and all the
relevant experimental parameters used in implementation for the proteins GR2,
ubiquitin and ER75.

Table 7. Acquisition Parameters for GFT NMR Experiments Recorded for the 7 kDa Protein GR2.

	(4,3)D HNNCACBCA	(4,3)D CBCACA(CO)NHN	(5,3)D HBHACBCACA(CO)NHN	(5,3)D HCC,CH-COSY
^{1}H Resonance Frequency	600 MHz	600 MHz	600 MHz	600 MHz
No. of Points[a] (t_1, t_2, t_3)				
Collected:	64, 24 512	64, 24 512	64, 24 512	100, 22, 512
After LP:	64, 24, 512	64, 24 512	64, 24 512	100, 22, 512
After Zero Filling	256, 64, 1024	256, 64, 1024	256, 64, 1024	256, 64, 1024
Window Functions[b]	sine 90/90/70	sine 90/90/70	sine 90/90/70	sine 90/90/70
No. of Transients[c]	2	2	2	2
Spectral Width[d] (ω_1, ω_2, ω_3; Hz)	12000, 1600, 6000	12000, 1600, 6000	25000, 1600, 6000	25000, 4500, 6000
t_{max}[e] (ms)	5.3, 15.0, 85.2	5.3, 15.0, 85.2	2.7, 15.0, 85.2	5.0, 4.0, 85.2
Carrier Position[f] (ω_1, ω_2, ω_3; ppm)	43.0, 120.4, 4.78	43.0, 120.4, 4.78	-1.0(^{1}H) /43.0(^{13}C) /56.3 (^{13}C), 120.4, 4,78	0.0(^{1}H)/ 43.0(^{13}C), 43.0, 4.78
Recycle Delay[g] (s)	0.7	0.7	1.0	0.7
Collection Time (hrs)[h]	6.5	6.5	32	22

5   a   "No. of Points" represents the number of complex data points used to sample indirect dimensions. Before Fourier transformation, the time domain points are extended by linear prediction (LP).

        b   The "Window Function" is a mathematical function multiplied with the FID along each indirect dimension before zero-filling and Fourier transformation.

10   c   "No. of Transients" represents the number of FIDs acquired for each real increment.

        d   "Spectral Width" is the frequency range covered in each dimension.

        e   "t_{max}" is the maximum chemical shift evolution time.

        f   "Carrier Position" refers to the frequency (in ppm) of the center point of the spectrum along each dimension.

15   g   "Recycle Delay" denotes the relaxation delay between acquisitions of FIDs.

        h   "Collection Time" is the total measurement time.

Table 8. Acquisition Parameters for GFT NMR Experiments Recorded for the 17 kDa Protein ER75.

	(4,3)D HNN<u>CACBCA</u>	(4,3)D HNN(CO)<u>CACBCA</u>	(5,3)D <u>HCC,CH</u>-COSY
Spectrometer	750 MHz	750 MHz	750 MHz
No. of Points[a] (t_1, t_2, t_3)			
Collected:	64, 32, 512	64, 32, 512	100, 24, 512
After LP:	64, 32, 512	64, 32, 512	100, 24, 512
After Zero Filling	256, 64, 1024	256, 64, 1024	256, 64, 1024
Window Functions[b]	sine 90/90/70	sine 90/90/70	sine 90/90/70
No. of Transients[c]	8	8	4
Spectral Width[d] (ω_1, ω_2, ω_3; Hz)	14000, 2200, 9000	14000, 2200, 9000	30000, 5400, 9000
t_{max} (ms)[e]	4.6, 14.1, 56.8	4.6, 14.1, 56.8	3.3, 4.4, 56.8
Carrier Position[f] (ω_1, ω_2, ω_3; ppm)	42.5, 117.2, 4.78	42.5, 117.2, 4.78	$0.0(^1H)/42.5(^{13}C)$, 42.5, 4.78
Recycle Delay[g] (s)	1.0	1.0	1.0
Collection Time (hrs)[h]	42	42	96

a   "No. of Points" represents the number of complex data points used to sample indirect dimensions. Before Fourier transformation, the time domain points are extended by linear prediction (LP).

b   The "Window Function" is a mathematical function multiplied with the FID along each indirect dimension before zero-filling and Fourier transformation.

c   "No. of Transients" represents the number of FIDs acquired for each real increment.

d   "Spectral Width" is the frequency range covered in each dimension.

e   "t_{max}" is the maximum chemical shift evolution time.

f   "Carrier Position" refers to the frequency (in ppm) of the center point of the spectrum along each dimension.

g   "Recycle Delay" denotes the relaxation delay between acquisitions of FIDs.

h   "Collection Time" is the total measurement time.

Table 9. Acquisition Parameters for GFT NMR Experiments Recorded for the 8.6 kDa Protein Ubiquitin

	(4,2)D <u>HCCH</u>-COSY	(5,3)D <u>HBCBCGCD</u>HD
Spectrometer	600 MHz	600 MHz
No. of Points[a] (t_1, t_2, t_3)		
Collected:	64, 1024	30, 10, 512
After LP:	64, 1024	30, 10, 512
After Zero Filling	256, 1024	256, 32, 1024
Window Functions[b]	sine 90/70	sine 90/90/70
No. of Transients[c]	4	4
Spectral Width[d] $(\omega_1, \omega_2, \omega_3; Hz)$	14000, 6794	8000, 2500, 6794
t_{max} (ms) [e]	4.5, 150.5	3.75, 4.0, 150.5
Carrier Position[f] $(\omega_1, \omega_2, \omega_3; ppm)$	4.78 (^1H) /125 (^{13}C), 4.78	4.78 (^1H) /38.0 (^{13}C)/125.0 (^{13}C), 135.0, 4.78
Recycle Delay (s)[g]	1.0	1.0
Collection Time (hrs)[h]	2.5	12

a "No. of Points" represents the number of complex data points used to sample indirect dimensions. Before Fourier transformation, the time domain points are extended by linear prediction (LP).
b The "Window Function" is a mathematical function multiplied with the FID along each indirect dimension before zero-filling and Fourier transformation.
c "No. of Transients" represents the number of FIDs acquired for each real increment.
d "Spectral Width" is the frequency range covered in each dimension.
e "t_{max}" is the maximum chemical shift evolution time.
f "Carrier Position" refers to the frequency (in ppm) of the center point of the spectrum along each dimension.
g "Recycle Delay" denotes the relaxation delay between acquisitions of FIDs.
h "Collection Time" is the total measurement time.

[0153]   Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing

from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.